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**Department of Forensic Science**

**VIRGINIA**

**DEPARTMENT**

**FORENSIC BIOLOGY SECTION**

**DATA BANK**

**PROCEDURES MANUAL**

**FLUORESCENT DETECTION**

**PCR-BASED STR**

**DNA PROTOCOL:**

**POWERPLEX<sup>®</sup> FUSION SYSTEM**

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## 1 ISOLATION OF DNA

### 1.1 Technical Notes

- 1.1.1 The QIAamp<sup>®</sup> extraction procedure uses spin columns to extract/purify DNA from buccal samples and dried blood stains. The QIAGEN<sup>®</sup> AL lysis buffer, included in the QIAamp<sup>®</sup> extraction kit, is a guanidine-based buffer. The guanidine helps to set up the binding conditions needed for the DNA to adhere to the spin column membrane. The QIAGEN<sup>®</sup> AL lysis buffer also contains a detergent to rupture leukocyte nuclear membranes which exposes the nucleic acids.
- 1.1.2 The QIAGEN<sup>®</sup> protease is similar to, but less stringent than ProK, and serves the same purpose for the breakdown of proteins into their constituent amino acids. The DNA yield reaches a maximum after lysis for 10 min at 56 °C. Longer incubation times have no effect on the overall yield of the purified DNA.
- 1.1.3 The QIAGEN<sup>®</sup> AW1 wash buffer is an ethanol-based stringent wash solution containing a low concentration of guanidine. This wash step removes any non-specific binding to the spin column membrane.
- 1.1.4 The QIAGEN<sup>®</sup> AW2 wash buffer is a Tris-based solution containing ethanol which will wash away any salts that are present.
- 1.1.5 The QIAGEN<sup>®</sup> AE elution buffer is a Tris-EDTA solution which elutes the DNA attached to the membrane and serves as a stable storage medium. Due to the high concentration of EDTA in this Qiagen-supplied reagent, all database samples will be eluted in Type I water to help prevent inhibition during the amplification process.
- 1.1.6 A random sample will be run with each set of convicted offender and arrestee sample extractions to serve as a verification that the samples are successfully entered into Combined DNA Index System (CODIS) and the search algorithm is working properly. This sample serves as an internal laboratory control and must be verified by the State CODIS Administrator or designee prior to the sizing data being considered acceptable. If a sample must be re-extracted a new random sample must be extracted along with the sample.
- 1.1.7 Routinely the QIAGEN<sup>®</sup> BioRobot<sup>®</sup> UNIVERSAL SYSTEM is used to isolate DNA from convicted offenders blood/buccal or arrestee buccal samples in accordance with the procedures manual. However, when necessary convicted offender blood/buccal and arrestee buccal samples may also be extracted using the manual QIAamp<sup>®</sup> Extraction Procedure.

### 1.2 Equipment

- Heat block or incubator, 56 °C
- Microcentrifuge
- Vortex mixer
- Tweezers
- Pipettes - 10 µL, 20 µL, 100 µL, 200 µL, 1000 µL
- Hole punch or scissors

### 1.3 Materials

- Microcentrifuge tubes, 1.5 mL
- Transfer pipettes
- Aerosol-resistant tips for pipettes, 10 µL, 20 µL, 100 µL, 200 µL, and 1000 µL
- QIAamp<sup>®</sup> spin columns
- QIAamp<sup>®</sup> 2-mL collection tubes

## 1.4 Reagents

- 95% Ethanol/Reagent Grade
- 1X phosphate-buffered saline (PBS), pH 7.2
- QIAGEN<sup>®</sup> protease
- QIAGEN<sup>®</sup> AL lysis buffer
- QIAGEN<sup>®</sup> AW1 wash buffer
- QIAGEN<sup>®</sup> AW2 wash buffer
- Type I Water

## 1.5 Manual QIAamp<sup>®</sup> Extraction Procedure—Arrestee and Offender Samples

- 1.5.1 Dried blood stains/buccal: Cut a 3 - 5 mm<sup>2</sup> blood stain/buccal using a hole puncher, punch one hole from the dried blood stain/buccal and transfer to a labeled 1.5 mL microcentrifuge tube. The scissors or hole punch should be cleaned between each punch using 10% bleach followed by isopropanol.
- 1.5.2 Add 180 µL PBS buffer to each tube and pulse spin to force the blood stain/buccal into the buffer. Incubate at 56 °C for ten minutes.
- 1.5.3 Add 20 µL of the QIAGEN<sup>®</sup> protease to each tube and vortex for 15 seconds to thoroughly mix the sample.

### WARNING!

**The QIAGEN<sup>®</sup> AL lysis buffer contains Guanidine Hydrochloride. This chemical will produce chlorine gas when mixed with bleach. When cleaning the counter tops after using the QIAGEN<sup>®</sup> extraction method wash the counter top first with water, then isopropanol, followed by a 10% bleach solution to disinfect the area.**

- 1.5.4 Add 200 µL of the QIAGEN<sup>®</sup> AL lysis buffer to each tube. Vortex the tube for 15 seconds to thoroughly mix the sample.
- 1.5.5 Incubate the tube in a 56 °C heat block for 10 minutes. Pulse spin before opening.
- 1.5.6 Add 200 µL 95% ethanol to each tube. Vortex the tube for 15 seconds to thoroughly mix the sample. Pulse spin before opening.
- 1.5.7 Transfer the supernatant from the tube to a labeled QIAamp<sup>®</sup> spin column that is inside of a 2.0 mL collection tube. Centrifuge the sample for 1 minute at 8,000 rpm.

NOTE: Be careful not to apply the sample to the rim of the spin column. Sample that has been deposited on the rim during the centrifugation will be transferred from the tube to the inside of the microcentrifuge.

- 1.5.8 Discard the 2.0 mL collection tube containing the filtrate and place the QIAamp<sup>®</sup> spin column into a clean 2.0 mL collection tube.
- 1.5.9 Add 500 µL of the QIAGEN<sup>®</sup> AW1 wash buffer and centrifuge for 1 minute at 8,000 rpm.
- 1.5.10 Discard the 2.0 mL collection tube containing the filtrate and place the QIAamp<sup>®</sup> spin column into a clean 2.0 mL collection tube.
- 1.5.11 Add 500 µL of the QIAGEN<sup>®</sup> AW2 wash buffer and centrifuge for 3.5 minutes at 13,200 rpm.
- 1.5.12 Discard the 2.0 mL collection tube containing the filtrate and place the QIAamp<sup>®</sup> spin column into a clean, labeled 1.5 mL microcentrifuge tube.

- 1.5.13 Add 50  $\mu$ L of Type I water to each QIAamp<sup>®</sup> spin column.
- 1.5.14 Incubate the sample at room temperature for 1 minute.
- 1.5.15 Place the 1.5 mL microcentrifuge tube containing the QIAamp<sup>®</sup> spin column into a microcentrifuge and spin for 1 minute at 8,000 rpm.
- 1.5.16 Discard the QIAamp<sup>®</sup> spin column and cap the 1.5 mL microcentrifuge tube containing the extracted DNA sample. Extracted DNA samples should be stored at -20°C.

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## 2 PCR AMPLIFICATION

### 2.1 Technical Notes

- 2.1.1 The PowerPlex® Fusion System contains 24 sets of primers and allows for the co-amplification and five-color detection of the 13 core CODIS (US) loci (CSF1PO, FGA, TH01, TPOX, vWA, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51 and D21S11), the 12 core European Standard Set loci (TH01, vWA, FGA, D21S11, D3S1358, D8S1179, D18S51, D10S1248, D22S1045, D2S441, D1S1656 and D12S391), Amelogenin, DYS391, Penta D, Penta E, D2S1338 and D19S433.
- 2.1.2 The PowerPlex® Fusion System provides all materials necessary to amplify STR regions of human genomic DNA, including a hot-start thermostable DNA polymerase, which is a component of the PowerPlex® Fusion 5X Master Mix.
- 2.1.3 The PowerPlex® Fusion System may be utilized with either extracted DNA (Qiagen) or as a direct amplification system for convicted offender/arrestee database samples.
- 2.1.4 A random sample will be run with each set of convicted offender and arrestee sample amplifications to serve as a verification that the samples are successfully entered into Combined DNA Index System (CODIS) and the search algorithm is working properly.
- 2.1.5 If a sample must be re-amplified, the random sample does not need to be re-amplified, providing that the random sample has typed correctly. The reagent blank also does not need to be re-amplified, provided the reagent blank is clear. However, the Control DNA (positive amplification control) and negative amplification control must be amplified with the re-amplified sample.

### 2.2 Equipment

- 9700 Thermal Cycler
- Pipettes - 2 µL, 10 µL, 20 µL, 100 µL, 200 µL and/or 1000 µL
- 8- Channel Pipette – Range 0.5 µL to 10 µL
- Repeat pipetter – Range 1.0 µL to 1.25 mL
- Biological Safety Hood
- Microcentrifuge tube racks
- 1.2 mm Harris Micro-Punch® and cutting mat or BSD® semi-automated punch system
- 70°C heat block

### 2.3 Materials

- Aerosol-resistant pipette tips - 10 µL, 20 µL, 100 µL, 200 µL and/or 1000 µL
- 1.5 mL microcentrifuge tubes
- 0.2 mL amp tubes or 96-well amplification plates and strip caps
- 96-well plate adhesive tape

### 2.4 Reagents

- PowerPlex® Fusion System 5X Primer Pair Mix (component of Fusion kit)
- PowerPlex® Fusion System 5X Master Mix (component of Fusion kit)
- Water, Amplification grade (component of Fusion kit)
- Control DNA (2800M positive amplification control) (component of Fusion kit)
- PunchSolution™ reagent (for direct amp use) (component of PunchSolution™ kit)
- 5X AmpSolution™ reagent (for direct amp use) (component of PunchSolution™ kit)

**NOTE:** A dedicated area, such as a biological hood or a separate room, should be used for preparing PCR amplification reactions. All equipment and supplies used to prepare amplification reactions should be kept in this dedicated "clean" area at all times. Do not use these items to handle amplified DNA or other

potential sources of contaminating DNA. Trace amounts of amplified DNA, if carried over into other samples before amplification, can lead to results that can be misinterpreted. DO NOT bring amplified DNA, or equipment and supplies used to handle amplified DNA, into the designated "clean" area. Wear clean disposable laboratory gloves while preparing samples for PCR amplification. Change gloves frequently or whenever there is a chance they have been contaminated with DNA.

## 2.5 PowerPlex® Fusion System Amplification Procedure for Extracted DNA (27 cycle half-volume reaction)

- 2.5.1 Calculate the required volume of each PCR amplification component to prepare a master mix by multiplying the volume by the number of samples. This should include a reagent blank, a random sample, positive and negative amplification controls and additional reaction volumes to compensate for any pipetting variation. The PCR Master Mix should be prepared in a clean 1.5 mL microcentrifuge tube.

Half Reaction Master Mix, 12.5 µL total volume, consists of:

2.5 µL PowerPlex® Fusion 5X Master Mix  
 2.5 µL PowerPlex® Fusion 5X Primer Pair Mix  
 7.5 µL DNA + Amplification grade Water

- 2.5.2 Add 5.0 µL of PCR Master Mix to each labeled tube or well of the 96 well plate using aerosol-resistant tips or repeat pipetter.

**NOTE:** If using a plate format, once all of the master mix is distributed, each column of the amplification plate should be covered with a strip cap. During the DNA transfer using the 8-channel pipetter, only one column should be opened at a time on both the extracted DNA plate as well as the amplification plate.

- 2.5.3 Prepare the sample DNA and controls as follows:

- 2.5.3.1 **Dried blood stains/buccal cells/random sample:** Add 1.0 – 7.5 µL of extracted DNA to the appropriately labeled sample tube or well. Bring the total DNA sample volume up to 7.5 µL by adding Amplification Grade Water to the tube or well.

**NOTE:** Weaker/difficult samples may require a higher volume to obtain a complete DNA profile.

- 2.5.3.2 **Positive Amplification Control:** Add 0.3 µL of the stock Control DNA to the appropriately labeled sample tube or well. Add 7.2 µL Amplification Grade Water to the tube or well.

- 2.5.3.3 **Negative Amplification Control:** Add 7.5 µL of amplification grade water to the appropriately labeled sample tube or well.

- 2.5.3.4 **Reagent Blank:** Add up to 7.5 µL of the reagent blank to the appropriately labeled sample tube or well. The volume added must match the maximum volume of DNA added to any sample in the amplification. Bring the total sample volume up to 7.5 µL by adding Amplification Grade Water to the tube or well.

- 2.5.4 Place the 96 well amplification plate or tubes into the 9700 thermal cycler. Move the thermal cycler lid over the samples and tighten.

- 2.5.5 **Extracted DNA:** Amplification Parameters for the 9700 Thermal Cycler:

- 2.5.5.1 96°C for 1 minute, then:

94°C for 10 seconds  
 59°C for 1 minute

72°C for 30 seconds  
For 27 cycles, then:

60°C for 10 minutes

4°C soak

The program must be run with Max mode as the ramp speed. The ramp speed is set after the thermal cycling run is started. The “Select Method Options” screen appears. Select “Max” for the ramp speed, and enter the reaction volume (13 µL)

**NOTE:** The PCR amplification program will run for approximately 1.5 hours.

2.5.6 Amplified DNA samples should be stored in the dark at -20 °C in a post-amp area.

## 2.6 PowerPlex® Fusion System Amplification Procedure for Direct Amp (25 cycle half-volume reaction)

2.6.1 Add 5.0 µL PunchSolution™ Reagent to each well of a 96-well plate that will contain a sample (including random sample and reagent blank). Do NOT add punch solution to wells that will ultimately contain the positive and negative amplification controls.

2.6.2 Punch samples using either 1.2 mm Harris Micro-Punch® and cutting mat or BSD® semi-automated punch system. Add 1-3 sample punches to the designated well.

**NOTE:** Weaker/difficult samples may require a higher number of punches to obtain a complete DNA profile.

**NOTE:** If a 1.2 mm Harris Micro-Punch® is used to manually place the sample punch in the well (i.e. for cotton swatch blood samples), then a witness must be used for the transfer.

2.6.3 After punching is completed, place the open plate into a 70°C heat block for 20 min. The sample wells should evaporate dry during this incubation.

**NOTE:** If the plate is carried to a different room for the incubation, cover the plate with adhesive tape while transporting it.

2.6.4 Calculate the required volume of each PCR amplification component to prepare a master mix by multiplying the volume by the number of samples. This should include a reagent blank, a random sample, positive and negative amplification controls and additional reaction volumes to compensate for any pipetting variation. The PCR Master Mix should be prepared in a clean 1.5 mL microcentrifuge tube.

Half Reaction Master Mix, 12.5 µL total volume, consists of:

- 2.5 µL PowerPlex® Fusion 5X Master Mix
- 2.5 µL PowerPlex® Fusion 5X Primer Pair Mix
- 2.5 µL 5X AmpSolution™ Reagent
- 5.0 µL Amplification grade Water

2.6.5 Prepare the sample DNA and controls as follows:

2.6.5.1 **Dried blood stains/buccal cells/random sample:** Add 12.5 µL of master mix to the appropriately labeled sample tube or well containing the punch.

2.6.5.2 **Positive Amplification Control:** Add 12.5 µL of master mix and 1.0 µL of the stock Control DNA to the appropriately labeled sample tube or well.

2.6.5.3 **Negative Amplification Control:** Do not add any additional volume after master mix.

2.6.5.4 **Reagent Blank:** Do not add any additional volume after master mix.

2.6.6 Place the 96 well amplification plate or tubes into the 9700 thermal cycler. Move the thermal cycler lid over the samples and tighten.

2.6.7 **Direct Amp:** Amplification Parameters for the 9700 Thermal Cycler:

2.6.7.1 96°C for 1 minute, then:

94°C for 10 seconds

59°C for 1 minute

72°C for 30 seconds

For 25 cycles, then:

60°C for 20 minutes

4°C soak

The program must be run with Max mode as the ramp speed. The ramp speed is set after the thermal cycling run is started. The “Select Method Options” screen appears. Select “Max” for the ramp speed, and enter the reaction volume (13 µL)

**NOTE:** The PCR amplification program will run for approximately 1.5 hours.

2.6.8 Amplified DNA samples should be stored in the dark at -20 °C in a post-amp area.

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### 3 CAPILLARY ELECTROPHORESIS

#### 3.1 Technical Notes

- 3.1.1 The Applied Biosystems 3500*xl* Genetic Analyzer is a fluorescent capillary electrophoresis (CE) instrument with a 24 capillary array which can be used for DNA sequencing and fragment analysis applications. This includes STR (Short Tandem Repeat) analysis as well as DNA sequencing and SNP (Single Nucleotide Polymorphism) analysis.
- 3.1.2 The capillary length and polymer type will vary based on the individual application. Sample handling and injection is automated by use of the autosampler, which holds two 96 well plates.
- 3.1.3 The polymer delivery pump (PDP) automates the replenishment of polymer between injections so that the instrument is capable of fully-automated operation.
- 3.1.4 Fluorescent detection is accomplished by utilizing single-line 505nm, solid-state long-life laser to excite fluorescent dyes and a CCD camera records the fluorescence emitted from samples within each of the 24 capillaries.
- 3.1.5 Data analysis including color separation is performed semi-automatically through the use of GeneMapper® *ID-X* software.
- 3.1.6 For the purpose of this manual, the following definitions apply:
- 3.1.6.1 Injection - all samples that were separated via CE at a single time. Thus, for one injection on the 3500*xl*, up to 24 samples can be separated at a single time.
- 3.1.6.2 Run - all samples that were defined on a single plate and loaded onto the instrument at a single time for separation, regardless of the number of injections utilized to separate each sample contained on the plate. Thus, a run could contain up to 96 samples for separation on a single plate.

#### 3.2 Equipment

- Applied Biosystems 3500*xl* Genetic Analyzer
- 96-well plate centrifuge
- Heat block, capable of 95 °C
- Pipettes - 2 µL, 10 µL, 20 µL, 100 µL, 200 µL and/or 1000 µL
- 8- Channel Pipette – Range 0.5 µL to 10 µL

#### 3.3 Materials

- 36-cm capillary array (Applied Biosystems, Inc. P/N 4404687 (24-capillary))
- Optical 96-Well Reaction Plate (Applied Biosystems, Inc. P/N N8010560)
- 96-well plate septum (Applied Biosystems, Inc. P/N 4315933)
- 96-well plate retainer and base (Applied Biosystems, Inc. P/N 4409530)
- Freezer block
- Aerosol-resistant tips for pipettes – 10µL, 20µL, 100µL, 200µL, and 1000 µL

#### 3.4 Reagents

- PowerPlex® 5-dye Matrix Standard 3100/3130 (Promega Corp. P/N DG4700)
- Hi-Di Formamide (Applied Biosystems, Inc. P/N 4311320)
- PowerPlex® Fusion Allelic Ladder (component of Fusion kit)
- CC5 Internal Lane Standard (ILS) 500 Size Standard (component of Fusion kit)
- POP-4 Polymer (Applied Biosystems, Inc. P/N 4393715 (384 samples) or P/N 4393710 (960 samples))

- Anode buffer container (Applied Biosystems, Inc. P/N 4393925)
- Cathode buffer container (Applied Biosystems, Inc. P/N 4408256)
- Cathode buffer septa (Applied Biosystems, Inc. P/N 4410715)

### 3.5 Safety Considerations

- 3.5.1 The Applied Biosystems 3500/3500xl Genetic Analyzer uses single-line 505nm, solid-state laser. All instrument panels must be in place on the instrument while the instrument is operating. When all panels are installed, there is no detectable radiation present. If any panel is removed when the laser is operating, (during service with safety interlocks disabled) you may be exposed to laser emissions in excess of the Class 3B rating. Do not remove safety labels or disable safety interlocks.
- 3.5.2 Performance Optimized Polymer (POP) is an irritant, always wear gloves when handling or cleaning liquid or dried polymer. It may cause skin, eye, and respiratory tract irritation.
- 3.5.3 Exposure to Hi-Di Formamide causes eye, skin, and respiratory tract irritation. It is a possible developmental and birth defect hazard. Read the SDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and nitrile gloves.

### 3.6 Instrument Preparation

- 3.6.1 Turn on the 3500xl instrument and the computer. The start-up order does not matter.
- 3.6.2 The 3500xl instrument will go through an initialization process.
- 3.6.3 On the computer, the Server Monitor/Daemon will launch automatically. The Server Monitor is completely loaded when the green check mark appears in the lower right corner of the screen.
- 3.6.4 After the Server Monitor/Daemon is fully loaded and there is a green light on the front of the 3500xl instrument, the 3500 Series Data Collection Software may be opened.
- 3.6.5 The Dashboard screen will launch (Figure 1).



Figure 1. The dashboard

3.6.6 Ensure the oven temperature is set to 60°C, and then select “Start Pre-Heat” at least 30 minutes prior to the first injection to preheat the oven.

3.6.7 Click “Refresh” in the “Consumables Information” window. The readings/gauges will be updated once the RFID tags are read.

3.6.7.1 Anode and cathode buffer containers will need to be changed if they have been on the instrument longer than fourteen days. See Appendix C for additional information regarding changing either buffer.

3.6.7.2 Verify that enough polymer remains in the pouch (# of samples remaining / # injections remaining). Also verify that the polymer has not been on the instrument longer than 14 days and that it has not expired. If the polymer is old, expired or an insufficient number remains, the polymer pouch will need to be changed prior to starting the run. See “Replenish polymer” in Appendix C for additional information.

3.6.8 Check the 3500xl instrument and perform the daily/pre-run tasks below.

TASK	FREQUENCY	FOR MORE INFORMATION
Visually inspect the level of fluid inside the ABC and the CBC. The fluid must line up with the fill line.	Before each run	Appendix C – changing ABC or CBC
Ensure that the plate assemblies are properly assembled.  <b>IMPORTANT!</b> Align the holes in the plate retainer with the holes in the septa to avoid damaging capillary tips.	Before each run	See section 3.7.2 and 3.7.3
Ensure that the plate assemblies and the CBC and septa are positioned on the plate deck properly. They should sit securely on the deck.	Before each run	
Check for bubbles in the pump block and channels.  <b>Note:</b> Use the Remove Bubble wizard to remove bubbles.	Daily or before each run	Appendix C – bubble remove wizard
Check the loading-end header to ensure that the capillary tips are not crushed or damaged. If crushed or damaged, the capillary array will need to be changed.	Daily or before each run	Appendix C – Change the Capillary Array
Clean the instrument surfaces of dried residue, spilled buffer, or dirt.	Daily	
Check for leaks and dried residue around the Buffer-Pin Valve, check valve, and array locking lever. If leaks persist, contact AB.	Daily	

### 3.7 Sample Preparation

3.7.1 To prepare a convicted offender/arrestee sample plate, prepare a ‘master mix’ of formamide and size standard as follows:

3.7.1.1 Thaw the CC5 ILS 500 and Hi-Di formamide. Vortex both briefly to mix.

3.7.1.2 Prepare a loading cocktail by combining and mixing CC5 ILS and Hi-Di formamide as follows:

**[(1.0ul CC5 ILS 500) X (# samples)] + [(10.0ul Hi-Di formamide) X (# samples)]**

NOTE: Be sure to add extra samples to account for pipetting variation which will vary depending on the number of samples. For example, if preparing an entire plate of samples (96), preparation of the master mix for approximately 106 samples is appropriate.

3.7.1.3 Vortex this 'master mix' to ensure the contents are thoroughly mixed.

3.7.1.4 Pipet 11  $\mu\text{L}$  of the 'master mix' into each used well of the 96-well plate.

3.7.1.5 10  $\mu\text{L}$  of Hi-Di formamide or extra 'master mix' should be pipetted into unused wells of the plate that will be injected to ensure that air is not injected into some of the capillaries. (That is, if one injection (24 samples total) will be completed on the 24-capillary 3500 $\mu\text{l}$ , it is necessary to ensure that all sample wells in all three columns contain liquid.)

3.7.1.6 Place the plate septum on top of the sample wells and use a scalpel to cut the septum into single column strips. During the amplified DNA transfer, only one column of both the CE plate as well as the amplification plate should be open at a time. Pipet PCR product (for samples, positive controls, and negative controls) into the corresponding well of the 96-well plate. Typically, 1  $\mu\text{L}$  of PCR product will be added. However, 0.5  $\mu\text{L}$  or 2  $\mu\text{L}$  of the PCR product may be used at the analyst's discretion and will be noted on the technical documentation.

NOTE: The same volume of PCR product (or more) used for the samples must also be used for the negative control(s) and reagent blank(s).

3.7.1.7 Pipet 1  $\mu\text{L}$  of allelic ladder into at least two wells per plate.

3.7.1.8 Cover wells with appropriate septa.

3.7.1.9 Centrifuge the plate briefly to remove air bubbles from all wells.

3.7.1.10 Just prior to loading the instrument, denature samples at 95  $^{\circ}\text{C}$  for 2 minutes, then immediately chill in a freezer block for 2 minutes.

3.7.2 Place the denatured and chilled plate into a blue plate base and snap the white plate retainer over the top taking care to align the tabs on both ends.

3.7.3 Press the tray button on the 3500 $\mu\text{l}$  to bring the autosampler tray forward. Open the instrument door and place the plate assembly into an open position (A or B) with the labeled end of the white plate retainer facing you.

3.7.4 Close the instrument door.

### 3.8 Instrument Operation

3.8.1 Importing a plate file

3.8.1.1 Click on "Library>plates" in the navigation pane.

3.8.1.2 Click "Import". Use the drop down menu to select "\*.xls". Browse to find the selected run file. Double click to import the correct file. Click "OK" after verifying the correct file was imported.

3.8.1.3 Open the imported file. Verify the information is correct and proceed to 3.8.3, "Linking plate for run".

## 3.8.2 Manually entering a plate file

3.8.2.1 Sample information may also be entered manually. In the Dashboard, click “Create New Plate” and enter the plate details.

- Enter the plate name
- Number of wells: 96
- Plate Type: HID
- Capillary Length: 36 cm
- Polymer: POP4
- Owner initials

3.8.2.1.1 Click on “Assign Plate Contents”

3.8.2.1.2 Using either the “Plate View” or “Table View”, enter the sample names in the appropriate well.

3.8.2.1.3 Under “Assays” click “Add from Library”. Select the appropriate file from the list and close the window. Data bank samples will be run with “PP\_Fusion\_24sec” and/or “PP\_Fusion\_12sec” or “PP\_Fusion\_24sec\_AND\_12sec”.

3.8.2.1.4 Under “File Name Conventions” click “Add from Library”. Select “Databank” from the list and close the window.

3.8.2.1.5 Under “Results Groups” click “Add from Library”. Select “Databank” from the list and close the window.

3.8.2.1.6 Highlight all samples to be run and click the check box in “Assays”, “File Name Conventions” and “Results Groups”.

3.8.2.1.7 In “Table View”, use the drop down under “Sample Type” to designate the Negative Control, Positive Control and Allelic Ladders. All other samples should remain “Sample”.

3.8.2.1.8 In the “UD2” (user defined comment) field, add the appropriate letters to indicate the specimen category.

3.8.2.1.9 Save the plate file and proceed to Section 3.8.3 “Linking Plate for Run”.

## 3.8.3 Linking plate for run

3.8.3.1 Click “Load Plates for Run” in the navigation pane.

3.8.3.2 Plates must be loaded onto the autosampler tray before it may be linked for a run. Click “Link Plate” in the appropriate position (A or B). Browse in the pop-up window to find the appropriate plate file. Double click on plate name to select, and then close the window.

3.8.3.3 Click “Start Run”. The instrument will notify you at this time if there is any instrument or reagent issue with the requested run. If an issue is detected, the run will not begin until the issue is resolved.

3.8.3.4 Alternatively, you may click “Create injection list”. Preview the injection list and move selected injections up or down to change the injection order. Once the order is determined, click “Start Run”. The injection order may also be changed once the run has begun with the exception of the first injection (which is running).

3.8.3.5 Following completion of the run, proceed to Chapter 4 for data analysis and interpretation.

### 3.8.4 Re-injections

3.8.4.1 Re-injections may be designated in two ways and are outlined below.

**NOTE:** The same injection time or longer than that used for the samples must be used for the negative controls and reagent blanks.

3.8.4.2 Re-injections may be designated on a plate while the plate is running on the instrument. After the run has started, in the “Monitor Run” screen, highlight the desired wells or select the desired injection to designate for re-injection.

3.8.4.2.1 To collect data for all wells in an injection, select the injection in the injection list. Click the “Re-inject” button.

3.8.4.2.2 To collect data for only specific wells, select the injection. Then select in the array view the capillary that corresponds to the well or sample of interest. Click the “Re-inject” button.

3.8.4.2.3 In the re-injection dialog box, select options, then click OK.

- The protocol to use for the re-injection: original, modified, new, or one from the library.
- When to make the injection.

3.8.4.2.4 If you select to re-inject a sample that includes an allelic ladder in its results group, but the allelic ladder is not part of the injection, the software prompts you to select one or more allelic ladder samples to re-inject.

3.8.4.2.5 In the “Add Allelic Ladder to Re-injection” screen, select one or more allelic ladder samples and then select whether to collect data for the remaining samples in the allelic ladder re-injection.

3.8.4.2.6 Then select whether to apply a modified instrument protocol to the allelic ladder re-injections, or whether to use the original instrument protocol for the allelic ladder re-injection(s). You will select the modified protocol in the next screen.

3.8.4.2.7 The injections will be designated on each well of the plate. Review for accuracy and change if necessary.

3.8.4.3 Alternatively, the plate file may be re-created using a different name. Typically re-injection files are named by appending “R” to the end of the plate file name. Select the “Assay” that designates the desired injection time.

3.8.4.3.1 If this option is chosen, the plate will need to be linked and run after the original plate run is completed.

3.8.4.4 To pause a run, click “pause” and the run will pause after the current injection completes.

3.8.4.4.1 “Resume” will resume the run.

3.8.4.5 To abort a run, click “abort” and the current injection immediately aborts and pauses the instrument run. You can resume the run or terminate the injection list. Do not click Delete to stop an injection.

3.8.4.6 To terminate an injection list, click “terminate injection list” and the instrument run will stop. Terminate is only active after you click “pause” or “abort”.

3.8.4.7 Following completion of the run, proceed to Chapter 4 for data analysis and interpretation.

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## 4 ANALYSIS AND INTERPRETATION OF POWERPLEX® FUSION SYSTEM CE DATA

### 4.1 Technical Notes

- 4.1.1 The PowerPlex® Fusion System is a 24-locus multiplex for human identification applications including forensic analysis, relationship testing and research use. This five-color system allows co-amplification and fluorescent detection of the 13 core CODIS (US) loci (CSF1PO, FGA, TH01, TPOX, vWA, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51 and D21S11), the 12 core European Standard Set (ESS) loci (TH01, vWA, FGA, D21S11, D3S1358, D8S1179, D18S51, D10S1248, D22S1045, D2S441, D1S1656 and D12S391) and Amelogenin for gender determination. In addition, the male-specific DYS391 locus is included to identify null Y allele results for Amelogenin. The Penta D and Penta E loci are included to increase discrimination and allow searching of databases that include profiles with these Penta loci. Finally, the D2S1338 and D19S433 loci, which are popular loci included in a number of databases, were incorporated to further increase the power of discrimination. This extended panel of STR markers is intended to satisfy both CODIS and ESS recommendations.<sup>1</sup>
- 4.1.2 The CC5 ILS 500 consists of 21 DNA fragments (60, 65, 80, 100, 120, 140, 160, 180, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, and 500 bp).
- 4.1.3 GeneMapper® *ID-X* (GMIDx) is an automated genotyping software program for all human identification (HID) data analysis needs, including forensic casework, databasing, and paternity testing. GMIDx software provides information on peak quality (height, base pair size, shape, number) as well as designating alleles.
- 4.1.4 The raw data obtained with the Applied Biosystems 3130x<sup>l</sup> and 3500x<sup>l</sup> Genetic Analyzers can be analyzed using GeneMapper® *ID-X* software.
- 4.1.5 Table 1 shows the PowerPlex® Fusion System STR Loci, Label, Chromosomal Location, and Repeat Sequence

STR Locus	Label	Chromosomal Location <sup>2</sup>	Repeat Sequence <sup>3</sup> 5'→3'
Amelogenin <sup>4</sup>	Fluorescein	Xp22.1-22.3 and Y	NA
D3S1358	Fluorescein	3p21.31 (45.557Mb)	TCTA Complex
D1S1656	Fluorescein	1q42 (228.972Mb)	TAGA Complex
D2S441	Fluorescein	2p14 (68.214Mb)	TCTA
D10S1248	Fluorescein	10q26.3 (130.567Mb)	GGAA
D13S317	Fluorescein	13q31.1 (81.62Mb)	TATC
Penta E	Fluorescein	15q26.2 (95.175Mb)	AAAGA
D16S539	JOE	16q24.1 (84.944Mb)	GATA
D18S51	JOE	18q21.33 (59.1Mb)	AGAA (19)
D2S1338	JOE	2q35 (218.705Mb)	TGCC/TTCC
CSF1PO	JOE	5q33.1 (149.436Mb)	AGAT
Penta D	JOE	21q22.3 (43.88Mb)	AAAGA
TH01	TMR-ET	11p15.5 (2.149Mb)	AATG (19)
vWA	TMR-ET	12p13.31 (5.963Mb)	TCTA Complex (19)
D21S11	TMR-ET	21q21.1 (19.476Mb)	TCTA Complex (19)
D7S820	TMR-ET	7q21.11 (83.433Mb)	GATA
D5S818	TMR-ET	5q23.2 (123.139Mb)	AGAT
TPOX	TMR-ET	2p25.3 (1.472Mb)	AATG
DYS391	TMR-ET	Y	TCTA
D8S1179	CXR-ET	8q24.13 (125.976Mb)	TCTA Complex (19)
D12S391	CXR-ET	12p12 (12.341Mb)	AGAT/AGAC Complex

D19S433	CXR-ET	19q12 (35.109Mb)	AAGG Complex
FGA	CXR-ET	4q28 (155.866Mb)	TTTC Complex (19)
D22S1045	CXR-ET	22q12.3 (35.779Mb)	ATT

**Table 1.** The PowerPlex® Fusion System STR Loci, Label, Chromosomal Location, and Repeat Sequence

<sup>2</sup>Information about the chromosomal location of these loci can be found in references 20, 21 and 22 and at:

[www.cstl.nist.gov/biotech/strbase/chrom.htm](http://www.cstl.nist.gov/biotech/strbase/chrom.htm)

<sup>3</sup>The August 1997 report of the DNA Commission of the International Society for Forensic Haemogenetics (ISFH) states, “1) for STR loci within coding genes, the coding strand shall be used and the repeat sequence motif defined using the first possible 5’ nucleotide of a repeat motif; and 2) for STR loci not associated with a coding gene, the first database entry or original literature description shall be used”.

<sup>4</sup>Amelogenin is not an STR but displays an 89-base, X-specific band and a 95-base, Y-specific band.

Table from Promega’s PowerPlex® Fusion System Technical Manual, revision 10/12

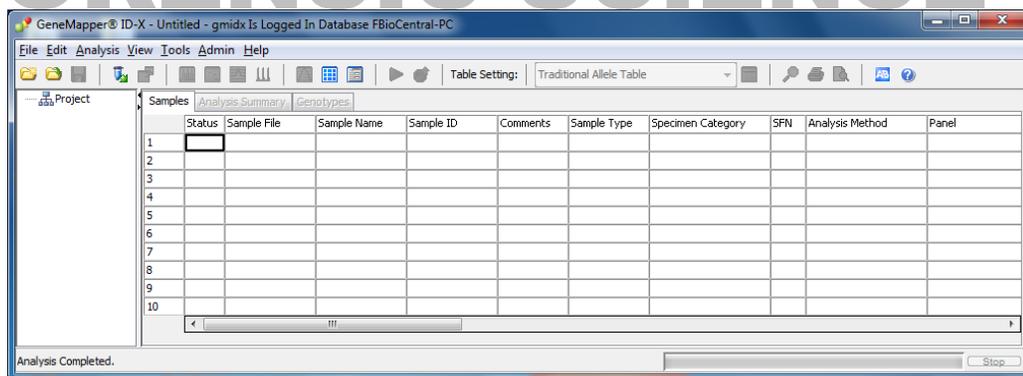
## 4.2 Logging On

4.2.1 Open the GeneMapper® ID-X (GMIDx) software by double-clicking on the software icon.

4.2.2 Select the appropriate user name and type the password. Click OK.

## 4.3 Project Window

4.3.1 Upon opening the software, the user is taken to the Project Window, as shown in Figure 1.



**Figure 1.** Project Window

4.3.2 To begin the data analysis, samples must be added to the project to be analyzed.

**NOTE:** All samples to be analyzed from a single run should be added at once. If not done in this manner, the GMIDx software may not associate the correct allelic ladders with the given samples.

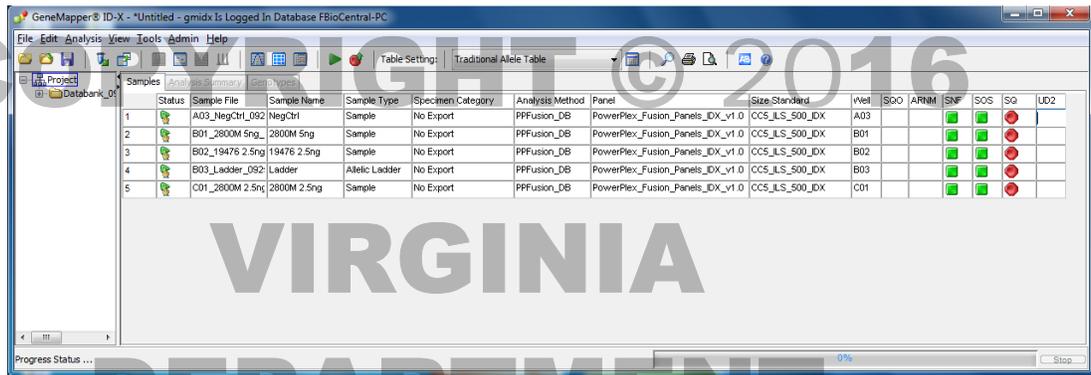
4.3.2.1 Choose “Edit” and “Add samples to Project”

4.3.2.2 Browse to the data files to analyze. You may select the entire folder or pick the files within the folder to analyze.

4.3.2.3 Click “Add to List” (bottom left of screen) then “Add” (bottom right of screen).

4.3.3 Upon addition of the samples to the project, the “Samples” tab will become populated, as shown in Figure 2. The population of the information is based on that entered in the Plate Map in the 3500xI Data

Collection Software. Double check to ensure that the correct panel, sample type, and analysis method are selected. If changes need to be made to this information, select the necessary cells and choose the appropriate settings from the drop-down menus. Verify that the information is correct before proceeding.



**Figure 2.** “Samples” tab upon adding samples to the newly created project

4.3.3.1 Analysis Method = “PPFusion\_DB”.

4.3.3.2 Panel = “PowerPlex\_Fusion\_Panels\_IDX\_v1.0”.

4.3.3.3 Size Standard = “CC5\_ILS\_500\_IDX”

4.3.4 Each data file has an  icon in the Status column indicating that it needs to be analyzed.

4.3.5 In the tree pane view on the left side of the screen, choose the file name of a negative control. Choose View>Raw Data to view the unseparated raw fluorescence data.

4.3.6 The recognizable pattern of the size standard should appear in orange. The raw data may be used to evaluate baseline problems or noise that could result in poor sizing. In addition, the start and stop points for the analysis should be noted. The start and stop points should be such that all size standards are encompassed by the data points analyzed. Typically, this is approximately 3,000 – 10,000 data points, but may need to be modified on a per-run basis, if, for example, at the 10,000 end point, the 500 bp size standard is missing. Extending the range beyond 10,000 will include the missing 500 bp fragment. This start and stop point may be edited in the Analysis Method Editor, as described below.

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## 4.3.7 Exit from the Negative Control raw data, and select Tools&gt;Analysis Method Editor

- 4.3.7.1 Click on the “Peak Detector” tab. The Peak Detector tab is where the values such as dye specific detection thresholds and analysis start and stop points are set (Figure 3).

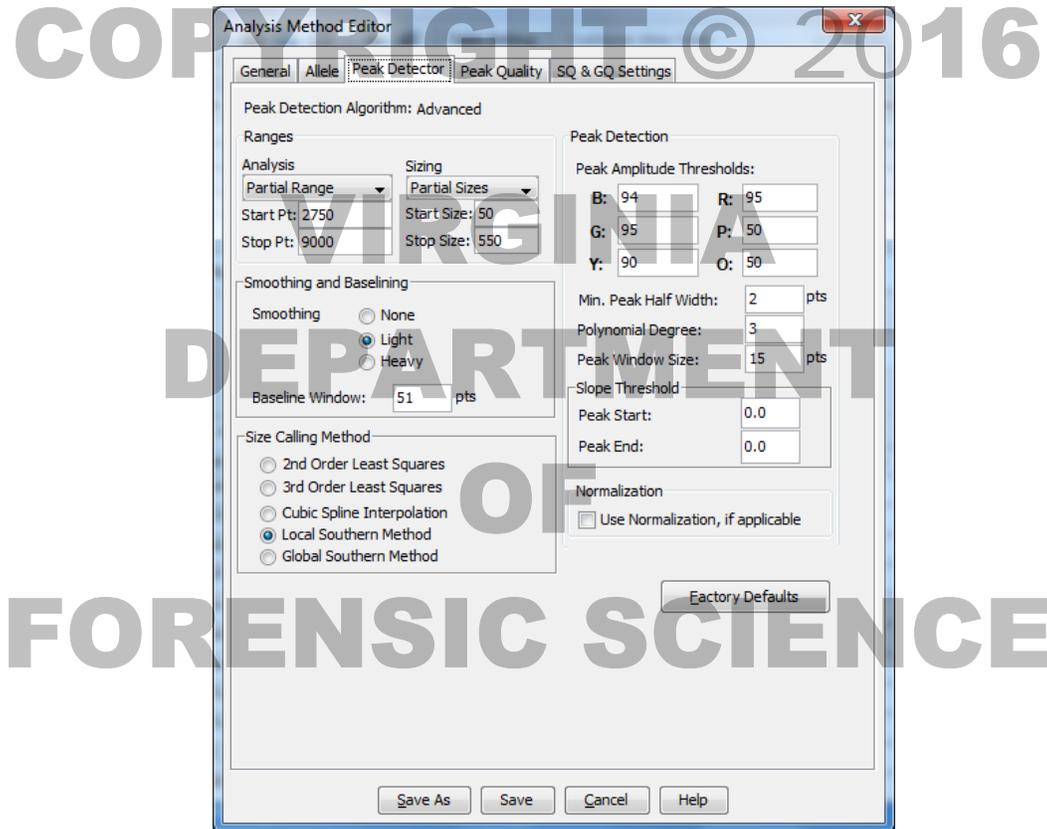


Figure 3. The Peak Detector Tab window

- 4.3.7.2 Change the analysis range to the start and stop points defined in 4.3.6, if necessary.
- 4.3.7.3 The values for the peak amplitude thresholds are based upon empirical data derived from the limit of detection analysis performed during the validation work and are averaged for both Data Bank 3500*x*l instruments. While the CC5 ILS 500 (which uses the orange dye) will have its default peak amplitude threshold set based on the LOD analysis, the threshold may be reduced when the ILS 500 peaks may fall below the default threshold (this can occasionally occur with the 12 second injection). It is important to note that ONLY the orange dye (CC5 ILS 500 size standard) may be adjusted if such conditions are present.

- 4.3.7.4 Smoothing should be set to “light” and the size calling method should be set to Local Southern Method.

**Note:** Sizing of Penta E and DYS391 alleles  $\geq 475$  bases will not use Local Southern Method. For Penta E, alleles  $>24$  will be labeled as “OL.”<sup>1</sup>

- 4.3.8 The Genotypes tab of the project window can display a number of additional “flags” to indicate the quality of the data. These may be used, at the analyst’s discretion, to aid in interpretation of the data. Refer to The GeneMapper® *ID-X* Software User’s Guide or the “Help” section in the software (Analysis Requirement Flags, Process Quality Value (PQV) Flags, and Data Review Flags) for an explanation of the usage of each flag.

- 4.3.9 Analyze the samples by clicking the green arrow:  (Analyze). Upon prompting, type a name for the project. Click OK.

#### 4.4 Examining Internal Lane Standard Results

- 4.4.1 The analysis run will finish and display the “Analysis Summary” screen which assesses run data according to Quality flag settings. Click on the “Samples” tab to view sample data. Assess the run by first examining the flags in the SQ (sizing quality) column. A green square indicates that a sample has passed the sizing criteria. For any samples that do not pass the sizing criteria, indicated by a yellow triangle or red octagon, assess the size standard by highlighting the samples and click on the icon:  or selecting Tools>Size Match Editor.

NOTE: If the majority of the size standards fail, look for a global problem with the run, such as a poorly-chosen sizing range in the Analysis Method Editor.

- 4.4.2 If the sizing standard is observed in the sample, check to see that all peaks are detected and the peaks are labeled correctly. The peaks should follow the pattern shown in Figure 4.

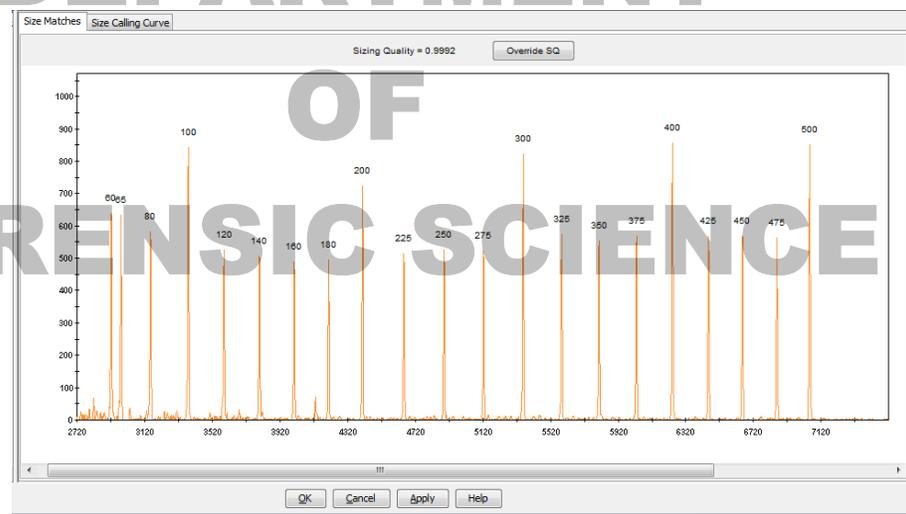


Figure 4. CC5 ILS 500 Size standard. The peaks are labeled with their base pair size.

- 4.4.3 The sizing quality of the sample is shown directly above the labeled size match electropherogram, as shown in Figure 4. Typically, a low sizing quality value (e.g., 0.5) is a result of a peak shift (the software incorrectly identifies one peak resulting in invalid positions for the other size standard peaks), a missing peak (the software does not identify a peak), or an extra peak (the software identifies a peak that is not a size standard peak, but a spike or pull-up peak).

- 4.4.3.1 If the size standard peak assignments are incorrect, adjust the peak assignments within the size match editor. To edit a particular size standard peak, right-click the peak to be edited to open the editing pop-up window and select “Add”, “Delete” or “Change” the selected peak. Move the cursor to the right to open the “Select Size” sub-menu. Choose the desired size from the menu.
- 4.4.3.2 When the peaks are sized properly, check the sizing quality by selecting Tools>Check Sizing Quality. If the peaks are sized correctly, the quality value will improve. If the peaks are sized correctly but the quality score is still below passing, click the “Override SQ” button, as shown in Figure 4, to set the sizing quality to 1.0. Note that the sizing quality will now read <1.0> and a blue “X” will appear in the Sample tab under the SQO (Sizing Quality Override) column to indicate the sizing quality has been overridden by the analyst. Alternatively, the sample may be re-injected at the analyst’s discretion.

### 4.5 Examining Allelic Ladder Results

4.5.1 In the “Samples” tab, select each allelic ladder. Display the plots by clicking the sample plots icon:



4.5.2 Verify that the allelic ladder is called correctly for each locus. Figure 5 represents an example of a PowerPlex® Fusion allelic ladder. Allelic ladder information is also contained in Table 2.

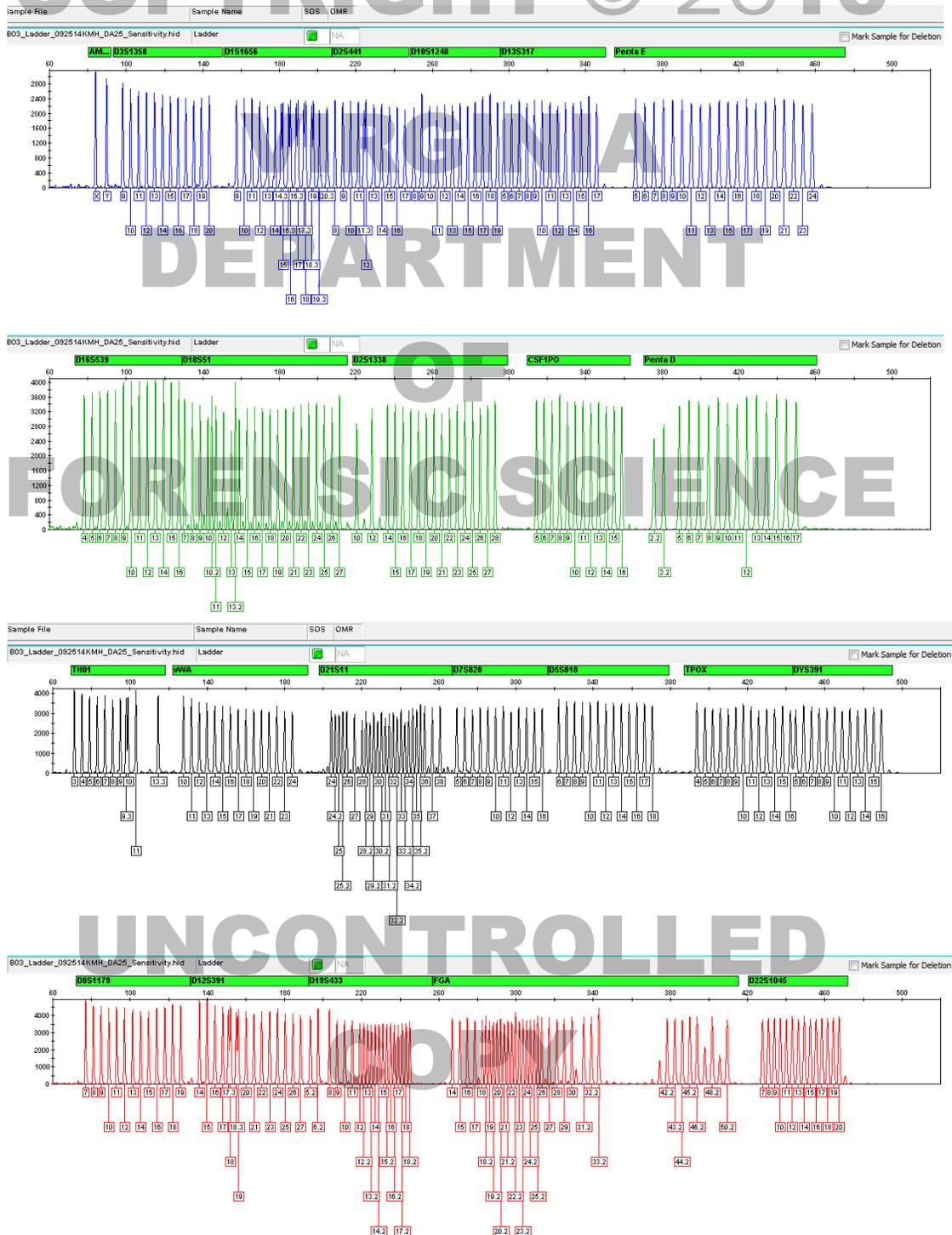


Figure 5. PowerPlex® Fusion Allelic Ladder

STR Locus	Label	Size Range of Allelic Ladder Components <sup>5,6</sup> (bases)	Repeat Numbers of Allelic Ladder Components <sup>7</sup>
Amelogenin <sup>8</sup>	Fluorescein	89, 95	X, Y
D3S1358	Fluorescein	103-147	9-20
D1S1656	Fluorescein	161-208	9-14, 14.3, 15, 15.3, 16, 16.3, 17, 17.3, 18, 18.3, 19, 19.3, 20.3
D2S441	Fluorescein	214-250	8-11, 11.3, 12-17
D10S1248	Fluorescein	256-280	8-19
D13S317	Fluorescein	302-350	5-17
Penta E	Fluorescein	371-466	5-24
D16S539	JOE	84-132	4-16
D18S51	JOE	134-214	7-10, 10.2, 11-13, 13.2, 14-27
D2S1338	JOE	224-296	10, 12, 14-28
CSF1PO	JOE	318-362	5-16
Penta D	JOE	377-450	2.2, 3.2, 5-17
TH01	TMR-ET	72-115	3-9, 9.3, 10-11, 13.3
vWA	TMR-ET	127-183	10-24
D21S11	TMR-ET	203-259	24, 24.2, 25, 25.2, 26-28, 28.2, 29, 29.2, 30, 30.2, 31, 31.2, 32, 32.2, 33, 33.2, 34, 34.2, 35, 35.2, 36-38
D7S820	TMR-ET	269-313	5-16
D5S818	TMR-ET	321-369	6-18
TPOX	TMR-ET	393-441	4-16
DYS391	TMR-ET	442-486	5-16
D8S1179	CXR-ET	76-124	7-19
D12S391	CXR-ET	133-185	14-17, 17.3, 18, 18.3, 19-27
D19S433	CXR-ET	193-245	5.2, 6.2, 8-12, 12.2, 13, 13.2, 14, 14.2, 15, 15.2, 16, 16.2, 17, 17.2, 18, 18.2
FGA	CXR-ET	265-411	14-18, 18.2, 19, 19.2, 20, 20.2, 21, 21.2, 22, 22.2, 23, 23.2, 24, 24.2, 25, 25.2, 26-30, 31.2, 32.2, 33.2, 42.2, 43.2, 44.2, 45.2, 46.2, 48.2, 50.2
D22S1045	CXR-ET	425-464	7-20

<sup>5</sup> The length of each allele in the allelic ladder has been confirmed by sequence analysis.

<sup>6</sup> When using an internal lane standard, such as the CC5 Internal Lane Standard 500, the calculated sizes of allelic ladder components may differ from those listed. This occurs because different sequences in allelic ladder and ILS components may cause differences in migration. The dye label and linker also affect migration of alleles.

<sup>7</sup> For a current list of microvariants, see the Variant Allele Report published at the U.S. National Institute of Standards and Technology (NIST) web site at: [www.cstl.nist.gov/div831/strbase/](http://www.cstl.nist.gov/div831/strbase/)

<sup>8</sup> Amelogenin is not an STR but displays an 89-base, X-specific band and a 95-base, Y-specific band.

Table taken from Promega's PowerPlex® Fusion System Technical Manual, revision 10/12

**Table 2.** The PowerPlex® Fusion System Allelic Ladder Information

#### 4.6 Examining the Reagent Blank

4.6.1 The reagent blank should be checked to ensure that no amplification product (called alleles) was observed. A called allele is a peak which fits the criteria to be designated an allele or OL (off ladder) and is above the allele calling threshold designated in the software and therefore is labeled.

4.6.1.1 If a signal (peak) is detected in the reagent blank at a single locus, the test results associated with the reagent blank will be evaluated against the samples around it to determine whether the result can be used.

4.6.1.1.1 If the reagent blank will be used as is, the Forensic Biology Program Manager or designee must grant approval. Documentation of this approval will be stored in the file.

4.6.1.1.2 Alternatively, if the reagent blank is re-injected or re-prepared and the result does not reproduce the signal (peak), the reagent blank result can be used without subsequent approval. The original sample injections can be analyzed if the reagent blank is clean upon re-injection.

4.6.1.1.3 If neither option above is utilized, then the reagent blank and all associated samples must be re-extracted and/or re-amplified.

4.6.1.2 If a signal (peak) is detected in the reagent blank at multiple loci, the test results for all loci will be considered inconclusive and all samples will be re-extracted and/or re-amplified.

4.6.2 For a typing result to be reported, the reagent blank must have been treated appropriately (i.e., same or longer injection times and same or larger volumes loaded into the injection cocktail).

#### 4.7 Examining the Negative Amplification Control

4.7.1 The negative amplification control should be checked to ensure that no amplification product (called alleles) was observed. A called allele is a peak which fits the criteria to be designated an allele or OL (off ladder) and is above the allele calling threshold designated in the software and therefore is labeled.

4.7.1.1 If a signal (peak) is detected in the negative amplification control at a single locus, the test results associated with the negative amplification control will be evaluated against the samples around it to determine whether the result can be used.

4.7.1.1.1 If the negative amplification control will be used as is, the Forensic Biology Program Manager or designee must grant approval. Documentation of this approval will be stored in the file.

4.7.1.1.2 Alternatively, if the negative amplification control is re-injected or re-prepared and the result does not reproduce the signal (peak), the negative amplification control result can be used without subsequent approval. The original sample injections can be analyzed if the negative amplification control is clean upon re-injection.

4.7.1.1.3 If neither option above is utilized, then the negative and all associated samples must be re-amplified.

4.7.1.2 If a signal (peak) is detected in the negative amplification control at multiple loci, the test results for all loci will be considered inconclusive and all samples will be re-amplified.

4.7.2 For a typing result to be reported, the negative control must have been treated appropriately (i.e., same or longer injection times and same or larger volumes loaded into the injection cocktail).

#### 4.8 Examining the Positive Amplification Control

4.8.1 If the positive control has injected poorly, it can be re-injected or if necessary, re-prepared and injected. The original sample injections can be analyzed if the correct types for the positive control are documented upon the re-injection or re-run.

- 4.8.2 The positive amplification control DNA must elicit the "known" genotype for each locus (see Table 3). If an allele is detected in the positive control DNA at a specific locus that is not consistent with the known genotype or no result is obtained at any locus, the samples will be re-amplified.

Locus	Genotype 2800M
Amelogenin	X,Y
D3S1358	17,18
D1S1656	12,13
D2S441	10,14
D10S1248	13,15
D13S317	9,11
Penta E	7,14
D16S539	9,13
D18S51	16,18
D2S1338	22,25
CSF1PO	12,12
Penta D	12,13
TH01	6,9.3
vWA	16,19
D21S11	29,31.2
D7S820	8,11
D5S818	12,12
TPOX	11,11
DYS391	10
D8S1179	14,15
D12S391	18,23
D19S433	13,14
FGA	20,23
D22S1045	16,16

**Table 3.** 2800M PowerPlex® Fusion profile

#### 4.9 Examining the Random Sample

- 4.9.1 The Random Sample profile, when searched in CODIS, must elicit the correct result. If the DNA sample number identified as a result of the search is different from the DNA sample number on the master list (maintained by the Forensic Biology Program Manager or designee) and is not a duplicate, all samples associated with the Random Sample will be re-extracted and/or re-amplified.
- 4.9.2 If the Random Sample has injected poorly, it can be re-injected or if necessary, re-prepared and injected. The original sample injections can be analyzed if the correct types for the Random Sample are documented upon the re-injection or re-run.
- 4.9.3 If no typing result is observed for the Random Sample (for convicted offender and arrestee sample analysis), all samples will be re-extracted and/or re-amplified.

#### 4.10 Examining Data Bank Samples

- 4.10.1 If it is determined that a sample contains stutter peaks at a majority of the loci and other artifactual peaks are visible throughout the electropherogram due to injecting too much and/or amplifying too much sample DNA, the sample may be re-injected using a reduced injection time and/or using less amplified DNA in the injection cocktail. Samples may also be re-amplified with less template DNA and then re-typed. If, however, the accurate profile can be determined from the original sample profile by both the analyst and the independent technical reviewer, then the data may be used.

- 4.10.2 All sample data must be reviewed carefully, with special attention paid to samples that display a yellow triangle or red octagon, indicating low quality analysis data.
- 4.10.2.1 A green square indicates that the data passed the initial (automated) analysis by the software in the respective category (such as sizing quality, off-scale data, etc.).
- 4.10.2.2 If a flag is yellow or red, it may be helpful to look at the raw data to determine the problem. To look at the raw data from the samples tab, highlight the sample and select View>Raw Data.
- 4.10.2.3 If it is determined that the sample should not be considered for interpretation due to problems within the raw data, the sample may be re-injected or run again using a different amplification reaction, more PCR product, less PCR product, or other necessary procedures.
- 4.10.2.4 If the sizing quality for a given sample is low, the sample will not be genotyped by the software.
- 4.10.3 Convicted offender and arrestee samples are expected to exhibit single source profiles. If more than one DNA profile is seen in a single sample, the sample will be re-extracted and/or re-amplified.
- 4.10.4 An independent electronic review must be performed for each sample and will be conducted by the technical reviewer using the GMIDx software.

#### 4.11 Identifying Amplification Artifacts

- 4.11.1 Since convicted offender and arrestee samples are not quantified prior to amplification, the overamplification of some samples will happen. When an excess of DNA is added to the amplification cocktail, artifacts may occur. Most of these artifacts will be below the 20% global filter cut-off used for convicted offender and arrestee samples and will not be labeled by the software. Application of the global filter causes all peaks below 20% of the highest peak within each marker not to be labeled. Figure 6 shows the settings contained in the “Allele” tab of the Analysis Method Editor.

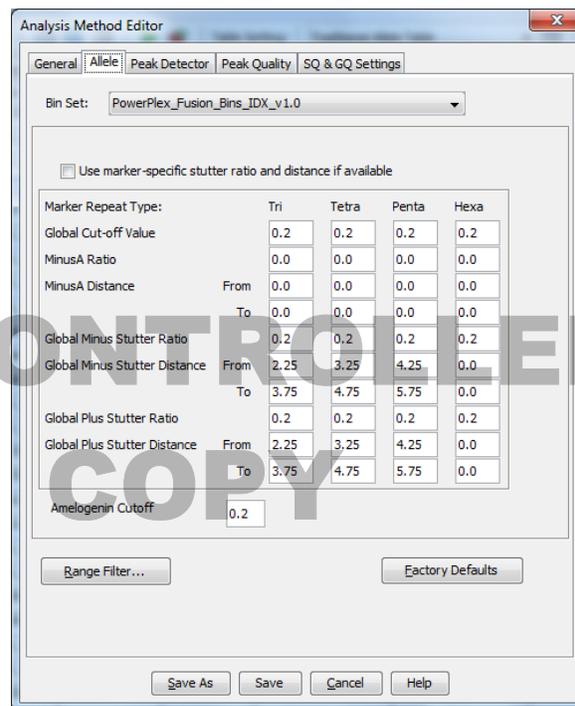


Figure 6. “Allele” tab of the Analysis Method Editor showing Global filter settings

4.11.1.1 For analysis of sample data, all electropherograms must be visually evaluated by the analyst to ensure that artifacts are not mislabeled. If a peak is mislabeled (e.g., stutter, spike, pull-up, etc.), the allele call can be electronically edited by the analyst.

4.11.1.1.1 To electronically edit the allele call of a mislabeled peak, select the peak of interest by right-clicking on it.

4.11.1.1.2 If the peak is already labeled, the allele call can be deleted. The user will be prompted to add a comment which will be maintained in the audit trail for the sample in GMIDx. For Data Bank convicted offender and arrestee samples, all deleted peaks will be considered to be “ART” and the display of the allele edit comment in the plot setting is not necessary.

4.11.1.2 If an analyst believes that a peak is real and the software does not label the peak due to the filter applied, the analyst may remove the filter and reanalyze the sample to better evaluate the peak in question.

#### 4.11.2 Off-scale data and Pull-up

4.11.2.1 Fluorescence intensity that exceeds the linear dynamic range for detection by the instrument is labeled "off-scale" data. Quantitation (peak height and area) for off-scale peaks is not accurate. An allele peak that is off-scale can cause the corresponding stutter peak to appear higher in relative intensity, thus increasing the calculated percent stutter.

4.11.2.2 Pull-up refers to peaks that are not true alleles but result from poor color separation of the raw data or off-scale data in one or more channels. Refer to Figure 7 for an example of pull-up at the D3S1358 locus.



**Figure 7.** Pull-up from the green channel (JOE) into the blue channel (FL), indicated by the red circle and shown as an edited “11” peak that has been deleted to indicate pull-up (artifact).

4.11.2.3 Recurrent pull-up due to poor color separation in samples indicates the need to complete a new spectral calibration.

## 4.11.3 Incomplete +A nucleotide addition

- 4.11.3.1 Terminal nucleotide addition occurs when the thermostable non-proofreading Taq DNA polymerase adds a nucleotide, generally adenine, to the 3' ends of amplified DNA fragments in a template-independent manner.<sup>1</sup> Modified primer sequences and the addition of a final extension step at 60°C to the amplification protocol are done to drive conditions to essentially complete terminal nucleotide addition when recommended amounts of template DNA are used. Excessive input DNA makes the adenine addition less efficient and thus PCR fragments shorter by one nucleotide than the amplicon size may appear (-A). Typically, these artifacts appear as a downstream shoulder on a peak.

## 4.11.4 Stutter

- 4.11.4.1 Repeat slippage, sometimes called “stutter” or “shadow bands”, is due to the loss of a repeat unit during DNA amplification, somatic variation within the DNA, or both.<sup>1</sup> Stutter peaks frequently appear during PowerPlex® Fusion System profiling. These are most commonly 4 nucleotides smaller than the amplicon size (true peak) for the tetranucleotide repeats, 3 nucleotides smaller for the trinucleotide repeats and 5 nucleotides smaller for the pentanucleotide repeats.
- 4.11.4.2 Stutter is typically more pronounced when excessive quantities of DNA are placed into the amplification cocktail. Stutter may also appear as multiples of the repeat unit (e.g. 8 nucleotides) or may be larger than the amplicon size (+4 stutter or “up-stutter”).

NOTE: Frequently, alleles with a greater number of repeat units will exhibit a higher percent stutter. A trinucleotide repeat locus, like D22S1045, will have more pronounced stutter in both n-3 and n+3 positions than a typical tetranucleotide repeat locus. The pattern and intensity of stutter may differ slightly between primer sets for the same loci.<sup>1</sup>

- 4.11.4.3 The GMIDx software is programmed to detect (i.e. not call as an allele) stutter peaks that are less than 20% of the largest peak at each locus.
- 4.11.4.3.1 If the ratio of the peak height for the smaller peak to the larger peak is less than 20%, the allele will be considered to be stutter by GMIDx and no allele will be designated even if it is suspected that the peak is a true allele.
- 4.11.4.3.2 If the ratio of the peak height for the smaller peak to the larger peak is above 20% (this event is generally observed in samples containing a high concentration of DNA or the DNA is partially degraded), the allele may still be called stutter once all loci have been taken into account and both the analyst and the independent technical reviewer are in agreement (based upon knowledge of the system and experience) that the smaller peak is stutter.

## 4.11.5 Spikes

- 4.11.5.1 Spikes are CE-related artifacts in which minor voltage changes or urea crystals passing by the laser can cause “spikes” or unexpected peaks. Spikes sometimes appear in one channel but often are easily identified by their presence in more than one channel.<sup>1</sup>
- 4.11.5.2 Signal spikes can frequently resemble peaks, but are characterized by their very narrow width. GMIDx has an algorithm that discriminates between true peaks and spikes, although, occasionally, the analyst may encounter a spike called as an allele. While the signal spike does not result from an actual DNA fragment, the software cannot always make this distinction and therefore calculates a size in base pairs for the spike.

4.11.6 Other artifact peaks can be observed at some of the PowerPlex® Fusion System loci. Low-level products can be seen in the n-2 and n+2 positions with some loci such as D1S1656, D13S317, D18S51, D21S11, D7S820, D5S818, D12S391 and D19S433. N-1 peaks are sometimes present at Amelogenin and D2S441. N-3 peaks are sometimes present at D12S391. Amplification-independent artifacts may be observed in template and non-template samples in the fluorescein (FL) channel at 64–65, 69–71 and 88–90 bases and in the JOE channel at 74–76 bases. Artifact peaks may be seen outside the locus panels in the FL channel at 70–74 bases, in the TMR-ET channel at 66–68 bases and in the CXR-ET channel at 58–65 bases. Artifacts that may be seen within the locus panels include allele 5 (84 bases) in D16S539 and peaks at 71–73 and 75–77 bases in TH01, 214 bases in D18S51 and 247 bases in D2S1338. These artifacts are typically below common minimum thresholds. (*Information taken from The PowerPlex® Fusion System Technical Manual, Promega, rev 10/12*)

#### 4.12 Microvariant/Off Ladder Variant Interpretation and Nomenclature

- 4.12.1 If an allele is labeled as “off ladder” or “OL”, careful review of the data should be conducted to determine if the allele is truly a microvariant/off-ladder allele or the allele is mislabeled (most commonly multiple alleles are mislabeled in that instance.)
- 4.12.1.1 The allelic ladders and size standard for the given sample should be reviewed for accurate calling. If multiple alleles are mislabeled, this is generally the cause.
- 4.12.2 If a true microvariant or other off-ladder allele is observed (and, if necessary on a sample by sample basis, confirmed by re-injection and/or re-amplification of the sample), the nomenclature described below will be used.
- 4.12.3 If the allele is not labeled by the GMIDx software, then the marker range must be changed so that the allele can be labeled. Approval for this change must be obtained from the Forensic Biology Program Manager or designee and documentation will be maintained in the plate file. Caution must be taken to ensure the marker range setting is restored to its original setting after the peak is labeled.
- 4.12.4 If any allele designation is changed from “OL” to a custom designation, then “FROM OL” must be entered into the Allele edit comment. Figure 8 below shows how the Plot Settings Editor>Labels>Custom allele must be set in order to display the allele edit comment for microvariants.

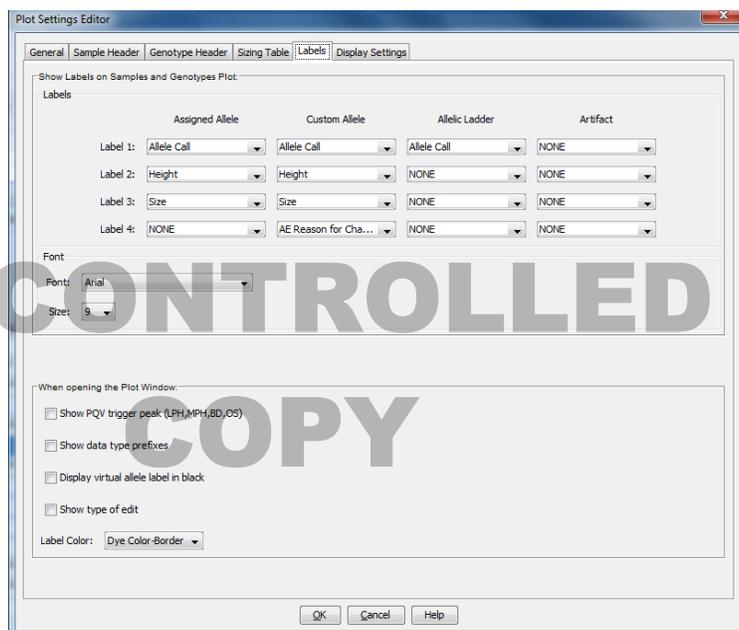


Figure 8. “Labels” screen of the Plot Settings Editor.

4.12.5 If an allele is seen in a region “off ladder” or OL alleles, i.e., larger than the largest allelic ladder peak of the largest molecular weight (MW) locus on the electropherogram, smaller than the smallest allelic ladder peak of the smallest MW locus on the electropherogram, or between loci, an allele designation based upon the nomenclature referenced below will be used.

4.12.5.1 If the allele is seen to the right (higher MW) of the largest allelic ladder peak of the largest MW locus, it will be assigned the type of the largest allele of the allelic ladder with a greater than sign (>).

4.12.5.2 If the allele is seen to the left of the smallest allelic ladder peak of the smallest MW locus, the allele will be assigned the type of the smallest allele of the allelic ladder with a less than sign (<). The "OL" value on the electropherogram will be manually changed to reflect the allele designation.

4.12.5.3 If an allele is seen between two loci and either the locus to the right OR left of the peak contains two peaks, the allele will be considered to belong with the locus not containing two peaks. The assignment of the allele designation will be based upon the nomenclature referenced below.

4.12.5.3.1 If the allele is to the right of the largest allelic ladder peak of the locus not containing two peaks, it will be assigned the type of the largest allelic ladder peak of the locus with a greater than sign (>). The "OL" value will be manually changed to reflect the allele designation.

4.12.5.3.2 If the allele is smaller than the smallest allelic ladder peak of the locus not containing two peaks, the allele will be assigned the type of the smallest allelic ladder peak of the locus with a less than sign (<). The "OL" value will be manually changed to reflect the allele designation.

4.12.5.4 If an allele is seen between two loci and neither the higher nor lower MW locus contains two alleles, follow the guidelines outlined below.

4.12.5.4.1 The base pair size for the allele in question will be compared to the base pair values for the largest allelic ladder peak of the lower molecular weight locus and to the smallest allelic ladder peak of the higher molecular weight locus.

4.12.5.4.2 An evaluation of the physical location of the allele in question with respect to the higher and lower molecular weight loci will be conducted.

4.12.5.4.3 An evaluation of the RFU values of the peaks in question may also help determine to which locus it belongs.

4.12.5.4.4 The allele in question will be considered to belong to the locus which is generally closest in proximity and falls within an appropriate size distance from the locus (i.e., one or two repeat units from the largest/smallest allelic ladder peak).

4.12.5.4.5 Once it has been determined to which locus the allele belongs, if the allele is larger than the largest allelic ladder peak it will be assigned the type of the largest allele of the allelic ladder with a greater than sign (>). If the allele is smaller than the smallest allelic ladder peak, the allele will be assigned the type of the smallest allele of the allelic ladder with a less than sign (<). The "OL" value on the electropherogram will be manually changed to reflect the allele designation.

4.12.5.5 If an allele is less than 500 bp in size, is visually between two allelic ladder peaks of the same locus, the GMIDx program generates a value “off ladder”. The allele will be considered to be

a microvariant. The allele will be assigned an allele designation of the lower repeat value followed by the number of bases in the incomplete repeat.

4.12.5.5.1 Example: an allele that migrates one base pair below the D16S539 14 allele will be designated as a D16S539 13.3. The "off ladder" value on the electropherogram will be manually changed to reflect the allele designation and the comment "FROM OL" will be added at the prompt.

4.12.5.5.2 To determine a microvariant allele call in the GMIDx software, highlight the sample in question and an allelic ladder under the "Samples" tab. Click on "View plots" and magnify the peak in question heavily. The ladder will also magnify automatically. Show bins, if needed, by clicking on "Bins" under the "View" header. The bin will appear as gray shading behind each peak. Compare the base pair size of the peak in question to the surrounding known ladder allele(s) to determine the size of the peak.

4.12.5.5.2.1 Microvariants not represented in the allelic ladder may be re-injected and/or re-amplified, if necessary, on a sample by sample basis in order to confirm the base pair size of the fragment.

#### 4.13 Gender

To indicate the gender of the contributor of a particular biological sample the Amelogenin locus may be used. A biological sample exhibiting a single peak at approximately 84 bp (X allele) will generally be considered to have originated from a female individual. A biological sample exhibiting a peak at approximately 84 bp (X allele) and a peak at approximately 90 bp (Y allele) will generally be considered to have originated from a male individual. The PowerPlex® Fusion system also contains the YSTR locus, DYS391, which may also be used for sex determination. If the Amelogenin result does not match the expected DYS391 result, the allele determination will be made on a case-by-case basis. The sample may be re-analyzed if deemed necessary.

#### 4.14 Peak Height Ratio

When a peak with a high rfu value and a peak with a low rfu value are observed within a single locus and the peaks are separated by greater than one repeat unit, the difference in peak height could be the result of the inability of the primer to perfectly hybridize to the template in the flanking region of one of the alleles. In order to determine if a DNA profile containing peaks with a difference in intensity is a result of primer mispairing or a mixture of biological material from more than one source, the analyst must take into account all of the loci, use experience and/or the heterozygous percent intensity values to make an informed decision. Databank samples are expected to be single source; however, an evaluation of the heterozygous percentage may be appropriate in instances where the sample may be contaminated.

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#### 4.15 File Transfer

If the project file must be transferred to another computer, the GMIDx project must first be exported.

- 4.15.1 Under the Tools drop-down window, select GMIDx Manager and the following window will open up (Figure 9).

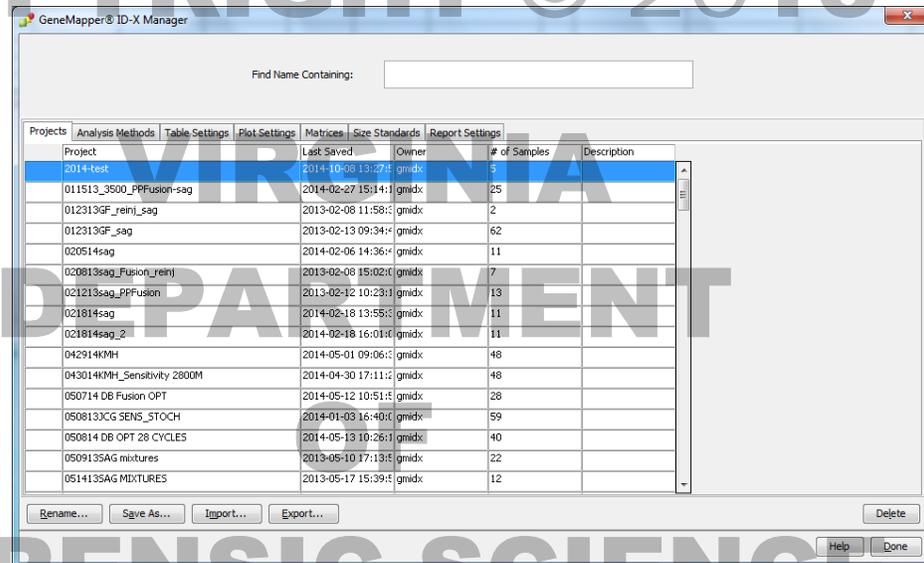


Figure 9. GMIDx Manager

- 4.15.1.1 Highlight the project file that is to be exported and select “export”. The project will be saved to the location and file folder designated. Transfer the project file to the appropriate medium/server for transfer to another computer workstation.
- 4.15.1.2 To open the project file at another computer workstation, open GMIDx and then open GeneMapper Manager as shown in Figure 7. Select “import” and browse to find the desired file. Select File>Open Project and find your project in the file list.

#### 4.16 Printing the Results

- 4.16.1 If a printed copy of the electropherogram is desired, after a review of each allele call in the samples plot, the x- and y-axes should be set accordingly for printing (as shown below). If the axis needs to be changed, right-click on the axis to display the scale settings. For printing purposes, the scale setting may be modified, if necessary, to allow visualization of specific characteristics of a particular electropherogram.

X-axis = approximately 60bp – 520bp  
 Negative Control or Reagent Blank: Y-axis = 0 – 100 RFU  
 Samples, Positive Control, and Allelic Ladders: Y-axis = scaled to largest peak

- 4.16.2 Print the electropherogram by File>Print.
- 4.16.3 Should additional documentation be necessary to support an allele designation, other views of the data (such as a zoomed-in view of an electropherogram) may be printed, at the analyst’s discretion.

#### REFERENCES

1. PowerPlex® Fusion System Technical Manual, Promega, rev. 10/12.

## APPENDIX A – CONVERSION FACTORS AND OTHER USEFUL INFORMATION

## I. CONVERSION FACTORS

One microgram =  $1 \mu\text{g} = 1 \times 10^{-6} \text{ g} = 1000 \text{ ng}$

One nanogram =  $1 \text{ ng} = 1 \times 10^{-9} \text{ g} = 1000 \text{ pg}$

One picogram =  $1 \text{ pg} = 1 \times 10^{-12} \text{ g} = 1 \times 10^{-3} \text{ ng}$

One liter =  $1 \text{ L} = 1000 \text{ mL}$

One milliliter =  $1 \text{ mL} = 1 \times 10^{-3} (0.001) \text{ L} = 1000 \mu\text{L}$

One microliter =  $1 \mu\text{L} = 0.001 \text{ mL} = 1 \times 10^{-3} \text{ mL} = 1 \times 10^{-6} \text{ L}$

## II. CONCENTRATIONS

## A. Concentration can be expressed several ways:

1. **Weight percent = (mass A/total mass of solution) x 100**

or simplified:  $\text{Wt \%}_A = (\text{g}_A/100 \text{ mL of solution}) \times 100$

2. **Volume percent = (volume A/total volume of solution) x 100**

or simplified:  $\text{Volume \%}_A = (\text{volume}_A/100 \text{ mL of solution}) \times 100$

3. **Molarity (*M*):  $M = \text{no. moles solute A/no. liters solution}$**

= molecular weight of solute A in 1000 mL solution

where 1 mole of A = 1 gram formula weight of A

4. **Normality (*N*) = no. MW/no. liters solution**

where in acid-base reactions:

MW acid = weight of acid which reacts with 1 mole of  $\text{OH}^-$

MW base = weight of base which reacts with 1 mole of  $\text{H}^+$

The normality of a given reagent depends on the reaction in which it participates. (Example: 1 L of 1M  $\text{H}_3\text{PO}_4$  which can have  $N = 1, 2$  or  $3$  depending upon the reaction in which it is involved.) Because of ambiguities, the concept of normality is to be used carefully.

## B. Examples:

1. **Prepare 50% solution of polyethylene glycol (PEG):**

Place 50 g of PEG in a flask and bring to a volume of 100 mL with  $\text{H}_2\text{O}$ .

$\text{Wt \% PEG} = (50 \text{ g}/100 \text{ mL}) \times 100 = 50\%$

2. Prepare 100 mL of 2M HCl from a stock solution of 12M HCl. (The question is how much stock solution of 12M HCl is needed?)

$$\text{Conc of Stock Soln} = C_s = 12M$$

$$\text{Conc of Final Soln} = C_f = 2M$$

$$\text{Volume of Stock Soln} = V_s = ?$$

$$\text{Volume of Final Soln} = V_f = 0.1 \text{ L}$$

$$C_s \cdot V_s = C_f \cdot V_f$$

$$V_s = (C_f \cdot V_f) / C_s = 2(0.1) / 12 = 0.2 / 12 = 0.0167 \text{ L}$$

$$V_s = 0.0167 \text{ L} = 16.7 \text{ mL}$$

Answer: Take 16.7 mL of 12M HCl and dilute to final volume of 100 mL to give 2M HCl.

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**APPENDIX B - REAGENTS**

1. This appendix describes the preparation of reagents necessary for the DNA analysis. For each reagent listed, the company and catalog number is included. As a reagent is prepared, it will be labeled to include the following information:

Identity  
 Concentration  
 Lot number  
 Date of preparation  
 Initials of preparer  
 Date of expiration

and if appropriate:

Date of autoclaving  
 Storage requirements

2. All reagents will be prepared with Type I water, unless otherwise stated.
3. All chemicals and reagents will be stored according to the manufacturers' specifications. All chemicals containing biologicals will be disposed of in biohazard bags. Unless otherwise stated, all other reagents used in Data Bank operations can be disposed of in the laboratory sink.
4. Any changes in chemical supply companies will be carefully checked by the Section Supervisor to ensure the chemical being provided meets the specifications necessary for the reagent. Any changes in chemical supply companies will be brought to the Forensic Biology Program Manager's attention so the list can be updated as necessary.
5. Concentrations preceding reagent components reflect the final concentrations of that specific component in the resulting mixture.
6. When a reagent is diluted and used by a number of analysts and retained, a lot number must be created and the reagent must be traceable to the reagent log. The container with the diluted reagent must contain the reagent stock lot number, the date the dilution was prepared, and the initials of the individual preparing the dilution. If a stock solution is diluted and used once and discarded or the dilution is used only by the analyst preparing the dilution, a new lot number does not need to be assigned or recorded in the reagent log. The original reagent stock lot number will be used.

**ANODE BUFFER CONTAINER**

Expiration date: Manufacturer's expiration date  
 Purchased from Applied Biosystems, Foster City, CA. PN 4393925

**CATHODE BUFFER CONTAINER**

Expiration date: Manufacturer's expiration date  
 Purchased from Applied Biosystems, Foster City, CA. PN 4408256

**CONDITIONING REAGENT**

Expiration date: Manufacturer's expiration date  
 Purchased from Applied Biosystems, Foster City, CA. PN 4409543

**ETHANOL, 95%**

Expiration date: Manufacturer's expiration date if listed, otherwise 3 years from date of receipt

**HI-DI™ FORMAMIDE**

Expiration date: Manufacturer's expiration date

Purchased from Applied Biosystems, Inc., Foster City, CA, PN 4311230, 25 mL each. Aliquot into 500 µL aliquots and store at -20°C.

**PERFORMANCE OPTIMIZED POLYMER – 4 (POP-4)**

Expiration date: Manufacturer's expiration date

Purchased from Applied Biosystems, Foster City, CA. PN 4393715 (384 samples) OR PN 4393710 (960 samples)

**PHOSPHATE-BUFFERED SALINE (PBS), 1X, pH 7.2**

Expiration date: Manufacturer's expiration date if listed, otherwise 3 years from date of receipt

**POWERPLEX® FUSION SYSTEM KIT**

Expiration date: Manufacturer's expiration date

Purchased from Promega, Madison, WI, Catalog Number DC2402 for 200 reaction kit or DC2408 for 800 reaction kit.

Kit components included (expiration date same as kit unless specified):

- POWERPLEX® FUSION 5X MASTER MIX
- POWERPLEX® FUSION 5X PRIMER PAIR MIX
- WATER, AMPLIFICATION GRADE
- 2800M CONTROL DNA, 10 ng/µl
- POWERPLEX® FUSION ALLELIC LADDER MIX
- CC5 INTERNAL LANE STANDARD 500

**POWERPLEX® 5-DYE MATRIX STANDARDS, 3100/3130**

Expiration date: Manufacturer's expiration date

Purchased from Promega, Madison, WI. Catalog Number DG4700.

**PUNCHSOLUTION™ KIT**

Expiration date: Manufacturer's expiration date

Purchased from Promega, Madison, WI. Catalog Number DC9271.

**QIAamp® DNA BLOOD MINI KIT**

Expiration date: Refer to individual kit component expiration dates.

Purchased from QIAGEN, Inc., Valencia, CA, Catalog number 51104 for the 50 sample kit OR 51106 for 250 sample kit.

Kit components include:

- QIAGEN® AL LYSIS BUFFER - Expiration date: Twelve months from date of receipt
- QIAGEN® AW1 WASH BUFFER (concentrate) - Expiration date: Twelve months from date of receipt. Dilute according to manufacturer's instructions before initial use.
- QIAGEN® AW2 WASH BUFFER (concentrate) - Expiration date: Twelve months from date of receipt. Dilute according to manufacturer's instructions before initial use.
- QIAGEN® AE ELUTION BUFFER – contained in the kit, but not used in Data Bank protocols
- QIAGEN® PROTEASE - Expiration date: 6 months after sample is reconstituted if stored at -20 °C, 2 months after sample is reconstituted if stored at 4 °C, or twelve months from date of receipt, whichever comes first. Reconstitute with supplied QIAGEN® protease solvent before initial use according to manufacturer's instructions.  
If the QIAGEN® Protease is purchased separately from the kit, then it is to be reconstituted with 7 mL of Type I water for each 125 mg bottle.
- QIAGEN® PROTEASE SOLVENT - Expiration date: Twelve months from date of receipt

**APPENDIX C - MAINTENANCE OF THE ABI 3500XL GENETIC ANALYZER**

1 The weekly and monthly maintenance requirements for the 3500xl are specified in Table 1.

<b>Task</b>	<b>Frequency</b>
Restart the computer and instrument	At each polymer change or as needed
Use a lab wipe to clean the anode buffer container valve pin assembly on the polymer delivery pump	Monthly or as needed
Run the Wash Pump and Channels Wizard (conditioning reagent)	Monthly or as needed
Flush the pump trap	Monthly or as needed
Empty the water trap waste container. The waste container is to the right of the pump block	Monthly or as needed
Replace cathode buffer septa	Monthly or as needed
Clean the autosampler and drip tray	Monthly or as needed
Remove dried polymer from the capillary tips with a lint-free wipe moistened with deionized water	Monthly or as needed
Defragment the hard drive	Monthly or before fragmentation reaches 10%

**Table 1.** Maintenance Tasks

1.1 Daily or pre-run tasks are specified in Section 3.6.8 of this manual

- 2 **Wizards** - Wizards are automated instrument processes which allow the user to perform a variety of tasks. Each wizard has been designed with specific instructions to achieve the purpose of the wizard. The wizards are accessed by selecting the desired wizard from the “Maintenance Wizards” menu as shown in Figure 1.

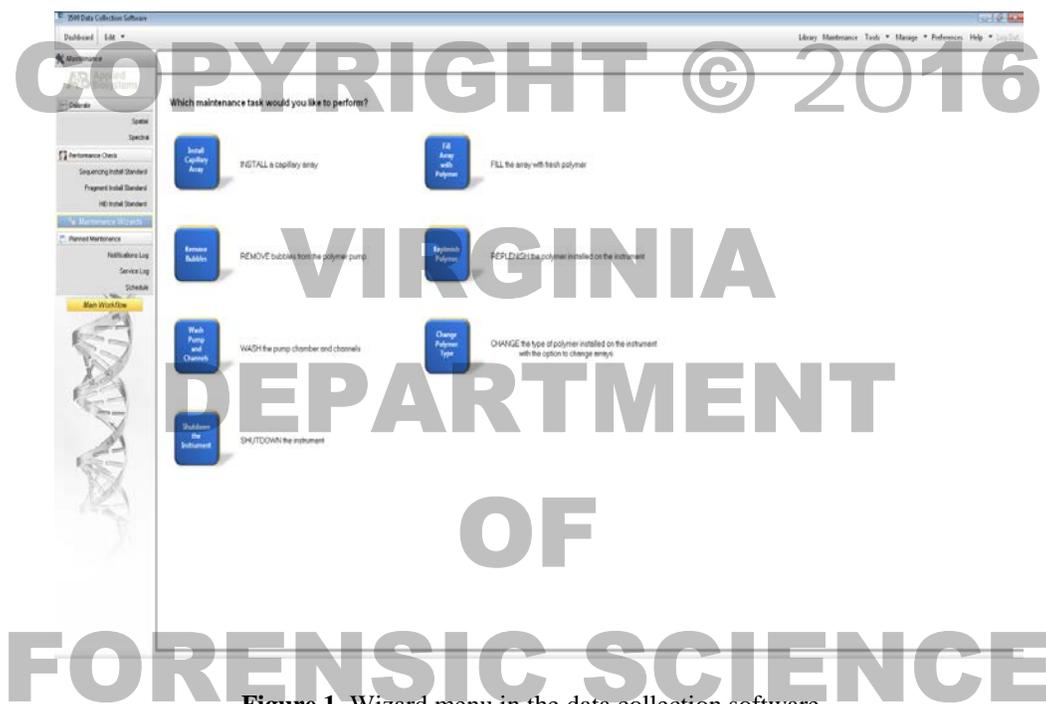


Figure 1. Wizard menu in the data collection software.

The wizards include:

### 2.1 Install a capillary array

- 2.1.1 Select Install Capillary Array wizard. Follow directions given in the wizard to change or install a capillary array. This wizard will take 15 to 45 minutes to complete.

### 2.2 Remove bubbles from the polymer pump

- 2.2.1 To remove bubbles from the polymer pump fluid path that travel from the polymer pouch through the pump, array port, and the anode buffer container, select Remove Bubbles wizard. Follow the directions given in the wizard. This wizard will take 5 to 15 minutes to complete.

### 2.3 Wash the pump chamber and channels

- 2.3.1 Conditioning reagent is used for priming the polymer pump, washing the polymer pump between polymer type changes, and during instrument shut down. Each pouch has adequate volume for a one-time use.
- 2.3.2 Select Wash pump chamber and channels wizard. Follow directions given in the wizard to wash the pump chamber and channels. The procedure takes about 25 minutes to complete.
- 2.3.3 Record the date of the conditioning reagent run, lot number and expiration of the reagent pouch in the appropriate QC log.

2.4 Fill the array with polymer

2.4.1 To fill the capillary array with the same type of polymer, in the Data Collection Software, select Maintenance Wizards>Fill the Array with fresh Polymer. Follow the directions given in the wizard.

2.5 Replenish the polymer installed on the instrument

2.5.1 Select Replenish Polymer Wizard. Follow the directions given in the wizard to load fresh polymer on the instrument. This wizard takes 10 to 20 minutes to complete.

2.5.2 Allow the new polymer pouch to equilibrate to room temperature prior to installing on the instrument. Verify that the polymer has not expired as the instrument WILL allow you to install polymer that is past the manufacturer's expiration.

2.5.3 Click "Refresh" on the dashboard to update the screen.

2.5.4 Record the date, lot number, and expiration date on the 3500xl Reagent Log.

2.6 Change the type of polymer installed on the instrument

2.6.1 All DFS DNA Data Bank validated procedures use POP4. This wizard will not be used.

2.7 Shutdown the instrument

2.7.1 For short-term and long-term shutdown, select Shutdown the instrument wizard. Follow the directions given in the wizard. This wizard takes 60 minutes to complete.

3 Restarting the computer

3.1 The computer and instrument will both be shut down and restarted each time the polymer is changed (Replenish polymer wizard). This action will be recorded on the 3500xl Reagent Log.

4 Defragment the hard drive

4.1 Go to Start>Programs>Accessories>System Tools>Disk Defragmenter and follow the prompts.

4.1.1 You can click "Analyze" to see if you should defragment or not.

5 Changing the Anode Buffer Container (ABC)

5.1 The lot number of the ABC is recorded in the raw data of each electropherogram. Verify that the buffer level is at or above the fill line and check that the seal is intact.

5.2 Invert the ABC, and then tilt it slightly to make sure most of the buffer is in the larger side of the container. There should be less than 1 ml of the buffer remaining in the smaller side of the container.

5.3 Peel off the seal at the top of the ABC and place the ABC into the Anode end of the instrument.

5.4 Click "Refresh" on the dashboard to update the screen.

6 Changing the Cathode Buffer Container (CBC)

6.1 The lot number of the CBC is recorded in the raw data of each electropherogram. Press the Tray button on the instrument to bring the old CBC forward. Remove the septas from the old container prior to discarding.

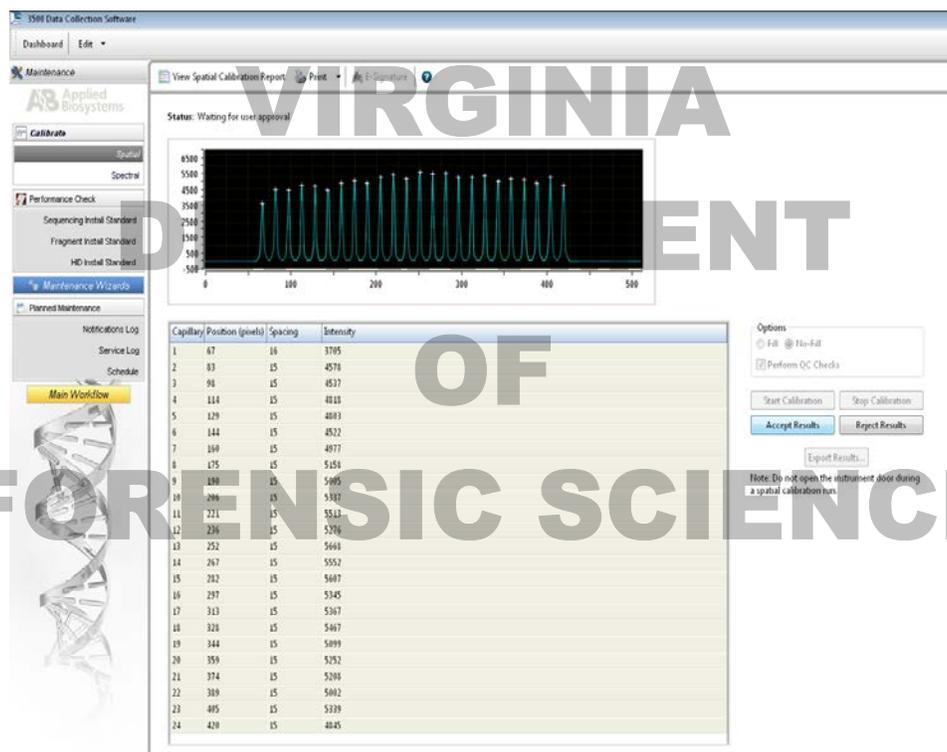
6.2 Verify that the buffer level is at or above the fill line and check that the seal is intact.

- 6.3 Tilt the CBC back and forth gently to ensure that the buffer is evenly distributed across the top of the baffles.
  - 6.4 Peel off the seal at the top of the CBC. Wipe off any buffer to make sure it is dry.
  - 6.5 Align and place the appropriate septa both sides. Push the septa lightly into the holes to start and then push firmly to seat the septa. Install the CBC on the autosampler.
  - 6.6 Click “Refresh” on the dashboard to update the screen.
- 7 Flush the water trap (pump trap)
- 7.1 The water trap must be flushed once per month to prolong the life of the pump and to clean any diluted polymer.
  - 7.2 Fill a 20mL, Luer lock syringe with distilled or deionized water. Expel bubbles from the syringe. Do not use a syringe smaller than 20mL as the pressure generated will be too great.
  - 7.3 Attach the syringe to the forward-facing Luer fitting at the top of the pump block. Hold the fitting with one hand while threading the syringe onto the fitting with the other hand.
  - 7.4 Open the Luer fitting by grasping the body of the fitting and turning it to loosen. Turn counterclockwise approximately one-half turn.
  - 7.5 DO NOT USE EXCESSIVE FORCE when you push the syringe plunger as this may damage the trap seals. Take approximately 30 seconds to flush 5 mL of water through the trap.
  - 7.6 Remove the syringe from the Luer fitting. Hold the fitting with one hand while turning the syringe counterclockwise with the other hand.
  - 7.7 Close the Luer fitting by lightly turning clockwise until the fitting seals against the block.
- 8 Performing a spatial
- 8.1 A spatial calibration is used by the instrument to correlate the signal from each capillary with the signal detected by the CCD camera. A spatial must be performed after any of the following events:
    - Replaced the capillary array
    - Opened the detector door or moved the detection cell
    - Moved the instrument
    - Service engineer performed an optical service procedure, such as realigned or replaced the laser or CCD camera or mirrors on the instrument.
  - 8.2 To access the Spatial Calibration screen, select Maintenance>Spatial Calibration in the navigation pane.
  - 8.3 Select “No Fill” or “Fill” to fill the array with polymer before starting the calibration.
  - 8.4 Check “Perform QC checks”. The software will check each capillary against the specified range for spacing and intensity.
  - 8.5 Click Start Calibration. The display updates as the run progresses.
  - 8.6 A Spatial QC Check error message is displayed if:
    - The average peak height or individual peak height is below the threshold
    - Uniformity or capillary spacing exceeds the threshold

8.7 When the run is complete, evaluate the calibration profile to ensure that you see:

- One sharp peak for each capillary. Small shoulders are acceptable
- One marker (+) at the apex of every peak. No off-apex markers.
- An even peak profiles (all peaks about the same height)

8.8 If the results meet the above criteria, click Accept Results. An example of a successful spatial profile is seen in Figure 2.



**Figure 2.** An example of an acceptable spatial calibration profile.

8.9 If the results do not meet the above criteria, click Reject Results, then go to “Spatial Calibration Troubleshooting” in the Applied Biosystems® 3500xl Genetic Analyzer user guide.

8.10 Only the most recent spatial calibration is maintained in the software. The software does not save historical calibration results.

## 9 Performing a spectral

9.1 The spectral calibration should be performed on dye set G5. Once generated, this file is applied during sample detection to calculate the spectral overlap between the five different dyes and separate the raw fluorescent signals into individual dye signals.

9.2 Perform a spectral for each dye set/polymer type combination you will use. A spectral must also be performed after any of the following events:

- Replaced the capillary array
- If a decrease is seen in spectral separation (pull-up in peaks) in the raw or analyzed data
- Service engineer performed an optical service procedure, such as realigned or replaced the laser or CCD camera or mirrors on the instrument

9.3 If you have not already done so, perform a spatial calibration.

9.4 Pre-heat the oven to 60°C. This is very important – preheating helps mitigate subtle first-run migration rate effects.

9.5 Then check consumables status on the dashboard. Ensure that:

- Consumables are not expired
- Adequate injections remain

9.6 Prepare the matrix standard for PowerPlex® Fusion

9.6.1 Remove the 5-dye matrix standards from the freezer, thaw, and vortex to mix thoroughly, but do NOT centrifuge.

9.6.2 **Initial dilution of concentrated fragments:** Dilute each of the concentrated dyes 1:10 with nuclease-free water (ex. 1 µL concentrated dye + 9 µL nuclease-free water). Vortex 5-10 seconds to mix.

9.6.3 **Fragment mix (using 1:10 dilutions of matrix standards):** Using these 1:10 dilutions, mix 7 µL of each diluted dye with 665 µL Hi-Di™ formamide for a total volume of 700 µL.

9.6.4 Vortex the formamide/matrix standard mixture thoroughly. Do not centrifuge the mixture.

9.7 Loading the samples on the 3500xl

9.7.1 Add 25 µL of the formamide/dye mix to each well of the first three columns of a 96-well plate.

9.7.2 After placing the septa on the plate, briefly centrifuge the plate to remove bubbles.

9.7.3 Denature the samples at 95°C for 3 minutes then immediately chill the plate in a freezer block for 3 minutes.

9.7.4 Place the plate in the plate base and cover with the plate retainer. Place the plate assembly in Position A on the autosampler with the label facing you.

9.8 Instrument Preparation

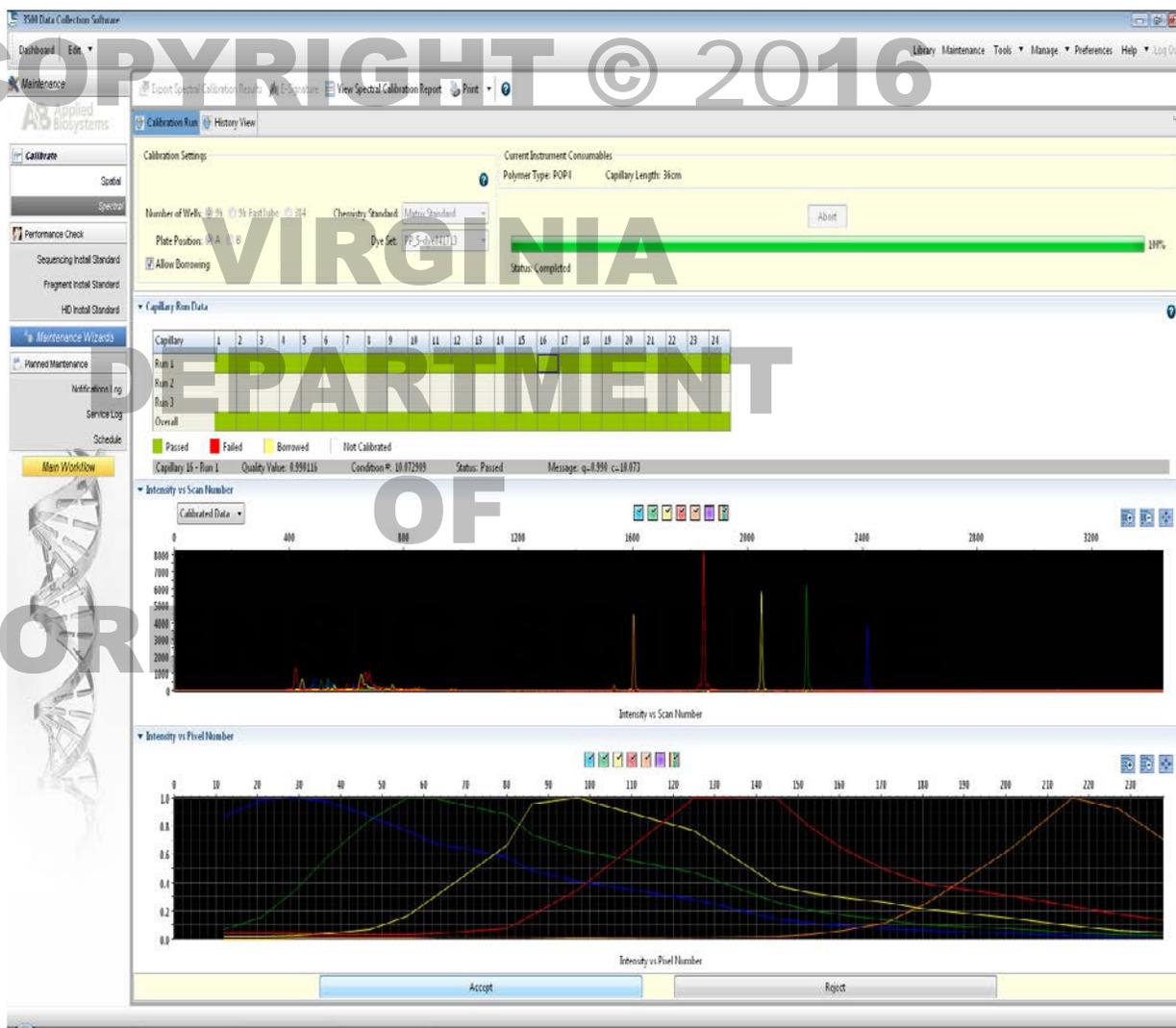
9.8.1 To perform the spectral calibration with the Promega 5-dye chemistry, go to the Maintenance tab, select “Spectral”, and under the Calibration Run tab, choose the appropriate fields in Calibration settings as indicated below:

- Number of wells: 96
- Chemistry standard: Matrix standard
- Plate Position: A
- Dye Set: PP 5-dye
- Allow borrowing: checked

9.8.2 Click Start Run.

## 9.9 Evaluating the Spectral calibration run

9.9.1 Figure 3 shows a complete and successful spectral run.



**Figure 3.** Completed spectral run

9.9.2 Passing and failing capillaries are shown in green and red, respectively. Borrowed capillaries are shown in yellow with an arrow indicating the adjacent capillary from which results were borrowed.

9.9.2.1 To display the result for each capillary (spectral data, Quality Value, and Condition Number) below the run results table, click a capillary in the table.

9.9.2.2 A spectral Quality Value reflects the confidence that the individual dye emission signals can be separated from the overall measured fluorescent signal. It is a measure of the consistency between the final matrix and the data from which it was computed. A Quality Value of 1.0 indicates high consistency, providing an ideal matrix with no detected pull-up/pull-down peaks.

9.9.2.3 A Condition Number indicates the amount of overlap between the dye peaks in the fluorescence emission spectra of the dyes in the dye set. If there is no overlap in a dye set, the Condition Number is 1.0 (ideal), the lowest possible value. The condition number increase with increasing peak overlap.

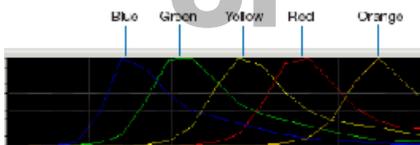
9.9.2.4 The PowerPlex® Fusion system uses the G5 dye set. Table 2 shows acceptable ranges.

Dye Set	Quality Value Minimum	Condition Number Maximum
AnyDye	0.8 (default)	20.0 (default)
E	0.95	5.5
E5	0.95	6.0
F	0.95	8.5
<b>G5</b>	<b>0.95</b>	<b>13.5</b>
J6	0.95	8.0
Z	0.95	5.5

**Table 2.** Acceptable condition number range and Q-values for analysis of spectral calibration data. Acceptable G5 dye set values are highlighted in yellow.

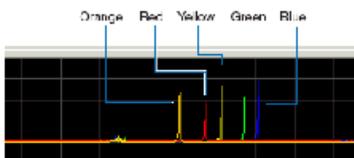
9.9.3 Examine the data for all capillaries before accepting the spectral calibration. Check that all capillaries meet the following criteria:

9.9.3.1 Order of the peaks in the spectral profile from left to right for 5-dye should be blue-green-yellow-red-orange as in Figure 4.



**Figure 4.** Order of peaks in the spectral profile

9.9.3.2 Order of the peaks in the raw data profile from left to right for 5-dye should be orange-red-yellow-green-blue as in Figure 5.



**Figure 5.** Order of peaks in the raw data profile

9.9.3.3 There should be no extraneous peaks in the raw data profile.

9.9.3.4 The peak morphology in the spectral profile should have no gross overlaps, dips or other irregularities. The peaks should be separate and distinct.

9.9.4 If any of the above criteria is not met, the calibration run has failed.

9.10 A spectral calibration can run up to three injections. The number of injections performed depends on the number of capillaries that pass or fail during an injection and whether the “Allow Borrowing” option is selected. If borrowing is allowed, then the capillaries can share information with adjacent capillaries within an injection or between injections. Up to three borrowing events are allowed.

9.11 Once all capillaries have been reviewed and accepted, click Accept.

9.12 If the results do not meet the above criteria, click Reject, then go to “Spectral Calibration Troubleshooting” in the Applied Biosystem’s® 3500xl Genetic Analyzer user guide.

9.13 Only the most recent spectral calibration is maintained in the software. The software does not save historical calibration results.

10 Long periods of inactivity

10.1 If the ABI 3500xl Genetic Analyzer remains unused for an extended period of time (approximately 2 weeks or longer), it is recommended that either a blank run (consisting of only formamide and size standard) be completed *or* the capillary array removed and the capillary ends stored in Type I H<sub>2</sub>O until further use.

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## APPENDIX D – STORAGE OF ELECTRONIC DATA

### Saving Sample files and Project files

- 1 Sample files and project files generated by the data bank using the Applied Biosystems, Inc. 3500*xl* Genetic Analyzer and analyzed using the GeneMapper® *ID-X* software will be saved onto a storage medium and stored according to the following procedures.
- 2 After the data has been technically reviewed and found to be acceptable, the electronic sample files and project files will be transferred onto a medium for permanent storage. The storage medium will be labeled appropriately to indicate the data files are PowerPlex® Fusion samples, the appropriate plate number, date and analyst's initials. The folders may be deleted from the computer after the data has been stored and a backup copy has been made.
- 3 The storage medium will then be stored securely with the data bank sample plate documentation in a specified location within the laboratory for easy retrieval.
- 4 If a file(s) needs to be subsequently added to the storage medium for a given plate of data bank samples, the analyst will make arrangements with his/her supervisor to have the storage medium retrieved from storage and have the file(s) added to the appropriate storage medium.

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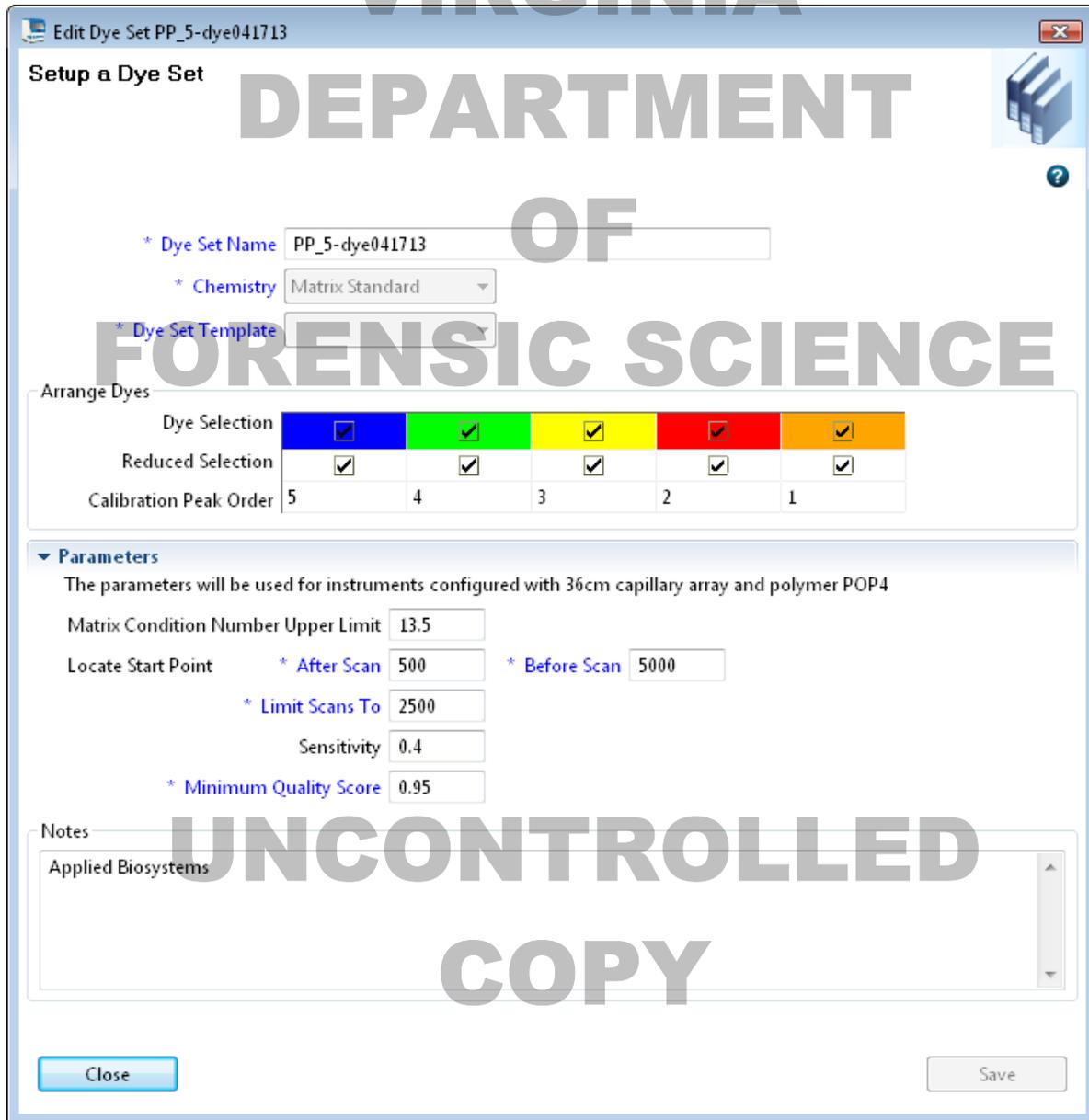
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**APPENDIX E – INITIAL SETUP OF GENEMAPPER® ID-X AND DATA COLLECTION SOFTWARE**

After installation of a new 3500xI instrument and installation of the GeneMapper™ ID-X software on a new computer, a number of customizations must be made. Displayed below are screen captures for settings in use by the 3500xI instruments and the GMIDx software. These settings may also be exported/imported from an established instrument/software to a new instrument/software.

For step-by-step directions for set up, the user guide for each system should be consulted (Applied Biosystem’s® 3500xI Genetic Analyzer User Guide, Applied Biosystems® GeneMapper® ID-X Software User Guide, and PowerPlex® Fusion System Technical Manual).

1 Table 1 shows settings in creating a dye set for the PowerPlex® Fusion system (3500xI).



**Table 1.** Initial dye set settings

2 Table 2 shows settings used in creating the 24 second Promega 5-dye instrument protocol (3500xl).

**Setup an Instrument Protocol**

Application Type:  Capillary Length:  cm Polymer:

Dye Set:

Instrument Protocol Properties

\* Run Module:   
 \* Protocol Name:   
 Description:

Oven Temperature (°C):  Run Voltage (kVolts):  PreRun Voltage (kVolts):  Injection Voltage (kVolts):   
 Run Time (sec.):  PreRun Time (sec.):  Injection Time (sec.):  Data Delay (sec.):

**Advanced Options**  
 Following values are not recommended to be changed.

Voltage Tolerance (kVolts):  Voltage # of Steps (nk):  Voltage Step Interval (sec.):   
 First Read Out Time (ms):  Second Read Out Time (ms):   
 Normalization Target:  Normalization Factor Threshold Min:  Normalization Factor Threshold Max:

**Table 2.** Initial 24 second instrument protocol settings.

3 Table 3 shows settings used in creating the 12 second Promega 5-dye instrument protocol (3500xl).

**Setup an Instrument Protocol**

Application Type:  Capillary Length:  cm Polymer:

Dye Set:

Instrument Protocol Properties

\* Run Module:   
 \* Protocol Name:   
 Description:

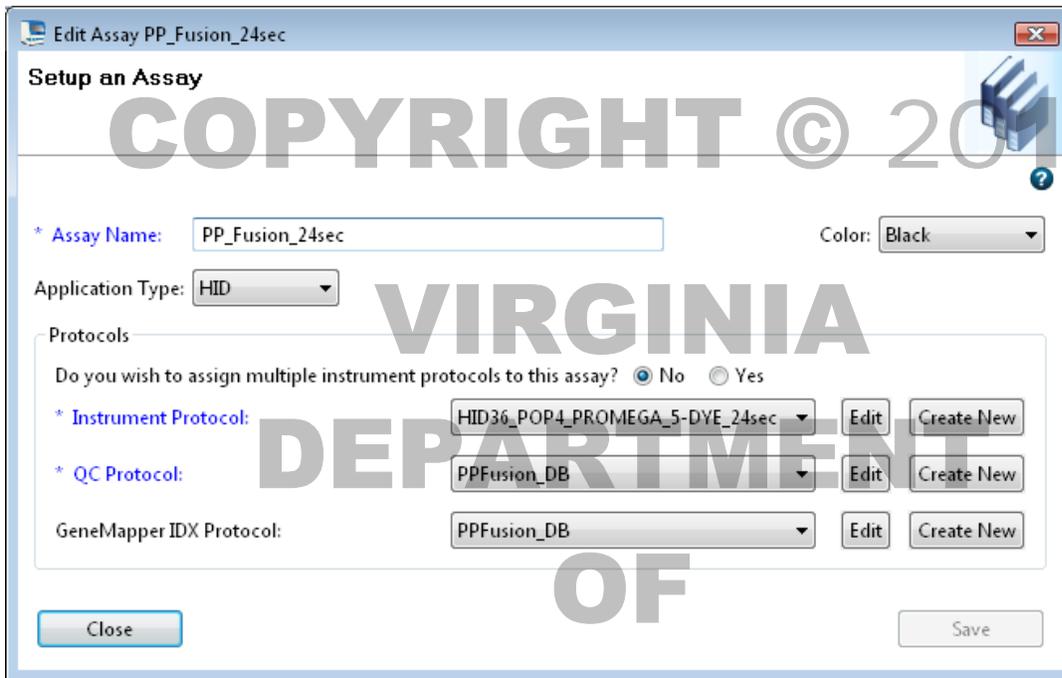
Oven Temperature (°C):  Run Voltage (kVolts):  PreRun Voltage (kVolts):  Injection Voltage (kVolts):   
 Run Time (sec.):  PreRun Time (sec.):  Injection Time (sec.):  Data Delay (sec.):

**Advanced Options**  
 Following values are not recommended to be changed.

Voltage Tolerance (kVolts):  Voltage # of Steps (nk):  Voltage Step Interval (sec.):   
 First Read Out Time (ms):  Second Read Out Time (ms):   
 Normalization Target:  Normalization Factor Threshold Min:  Normalization Factor Threshold Max:

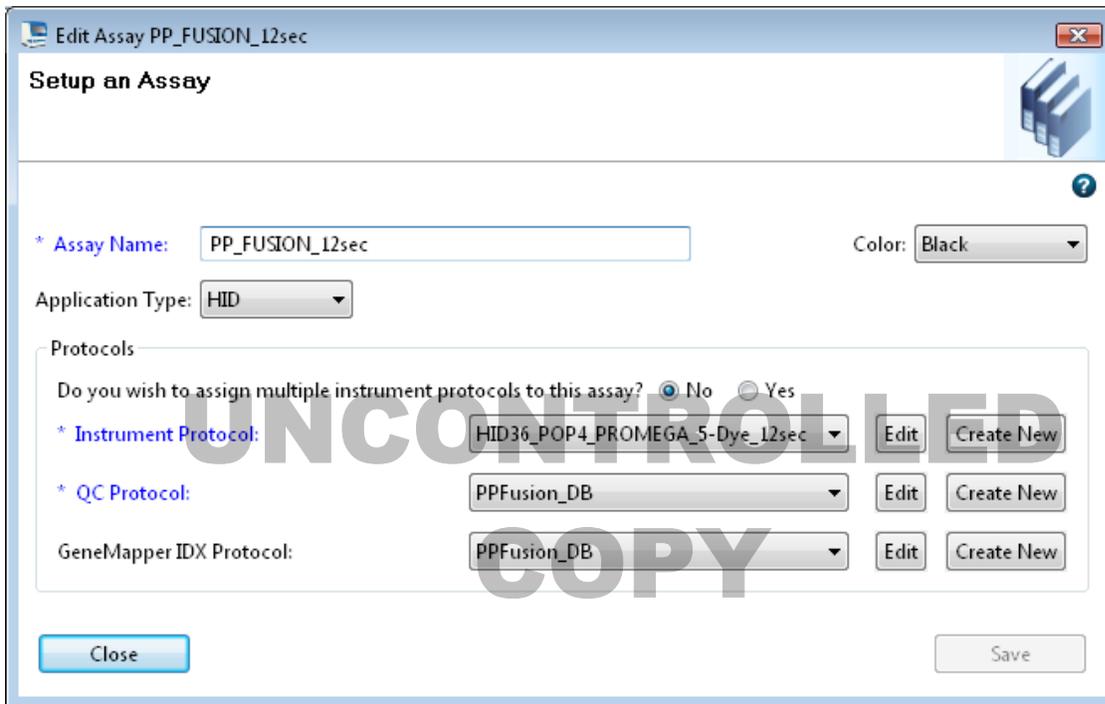
**Table 3.** Initial 12 second instrument protocol settings.

4 Table 4 shows settings used in creating the 24 second Fusion assay (3500xl)



**Table 4.** Initial 24 second assay settings.

5 Table 5 shows settings used in creating the 12 second Fusion assay (3500xl).



**Table 5.** Initial 12 second assay settings.

6 Table 6 shows settings used in creating the 12 and 24 second Fusion assay (3500x/).

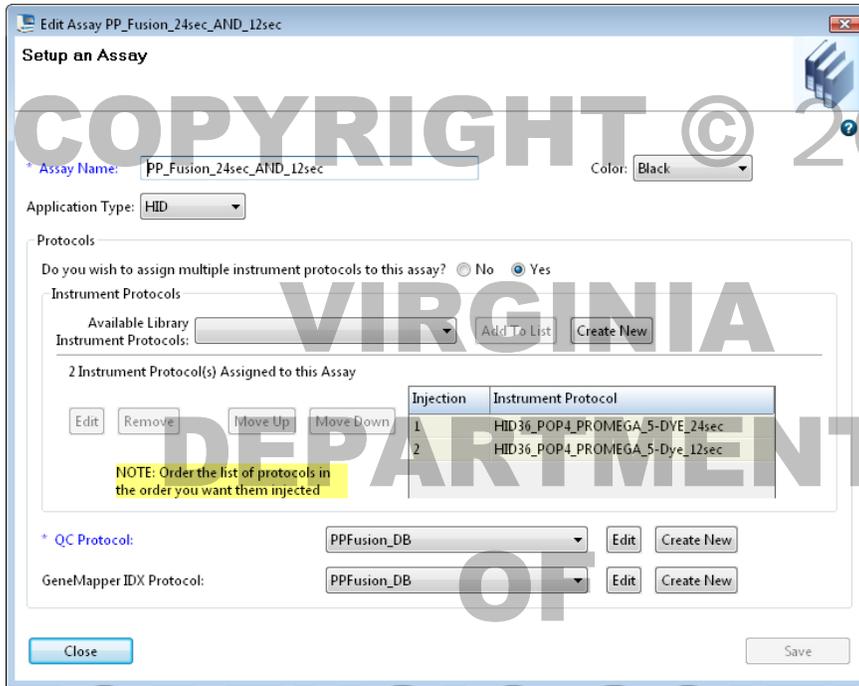


Table 6. Initial 12 and 24 second assay settings.

7 Table 7 shows settings used in creating a results group (3500x/).

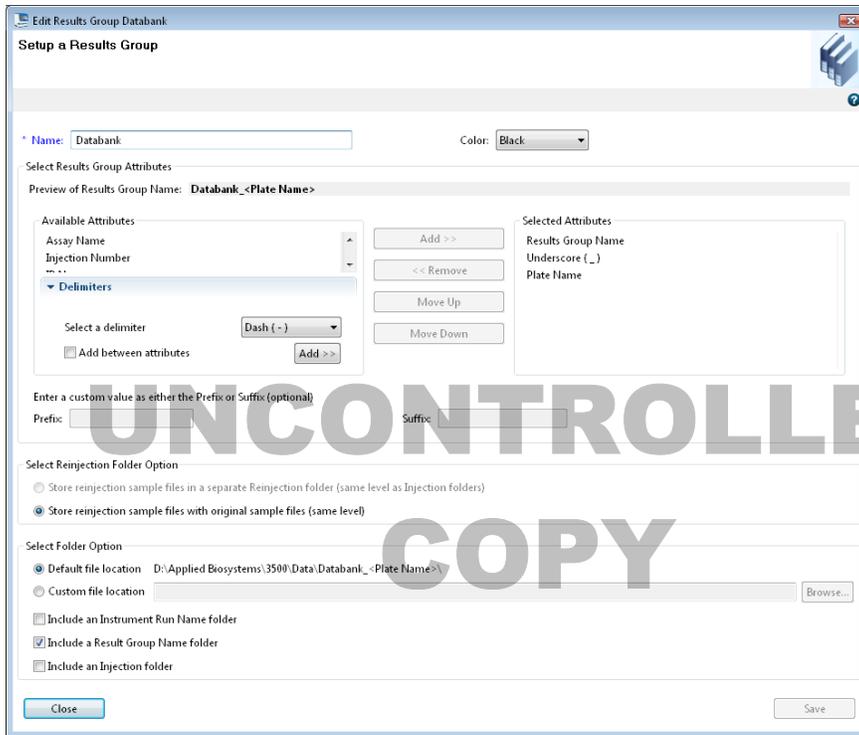
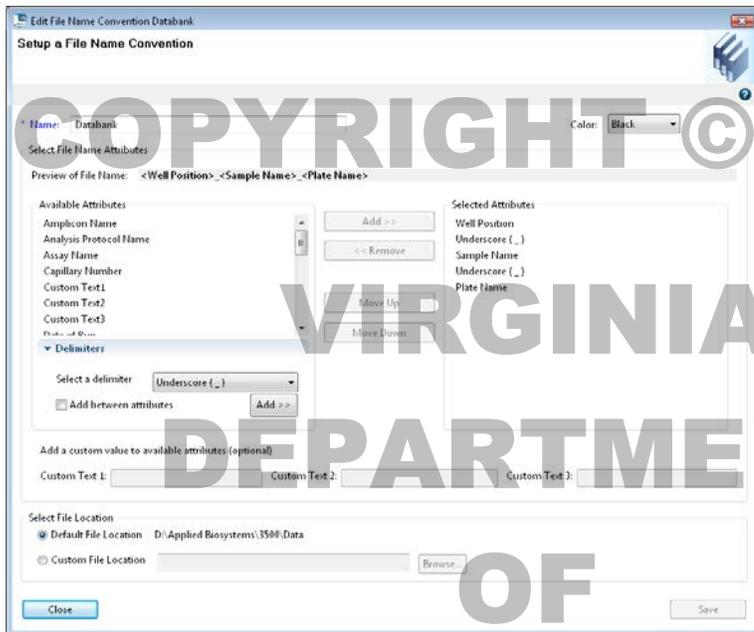


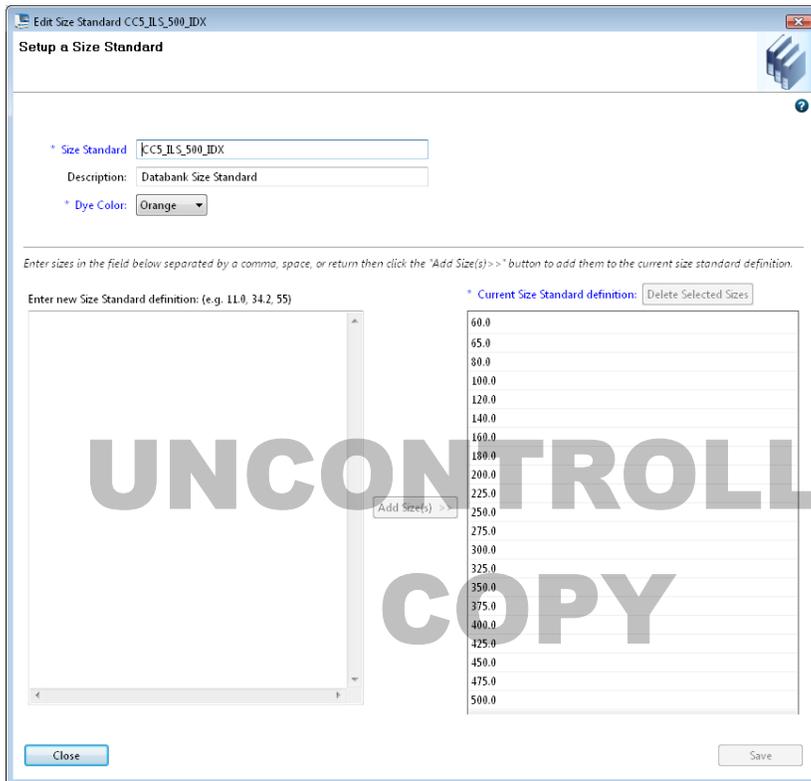
Table 7. Initial Data Bank results group settings.

8 Table 8 shows settings used in creating the file name convention (3500xI).



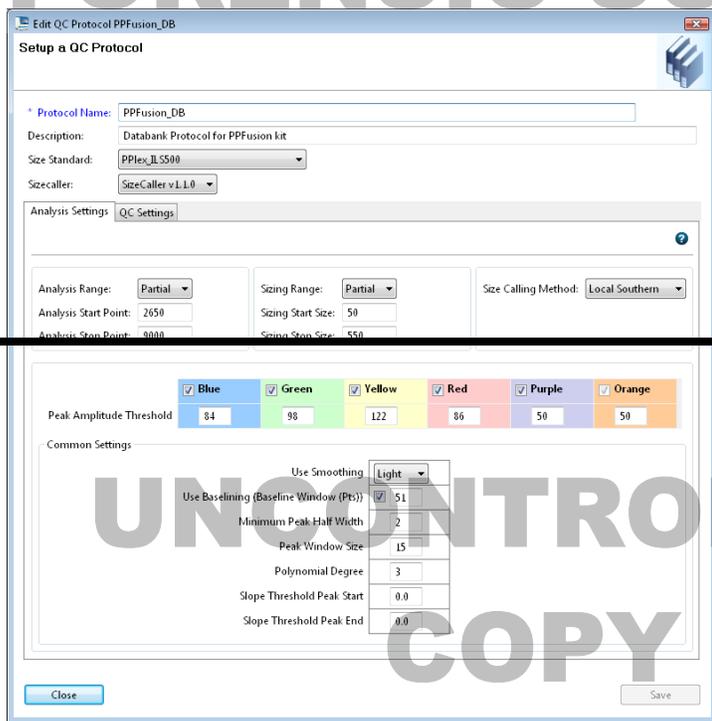
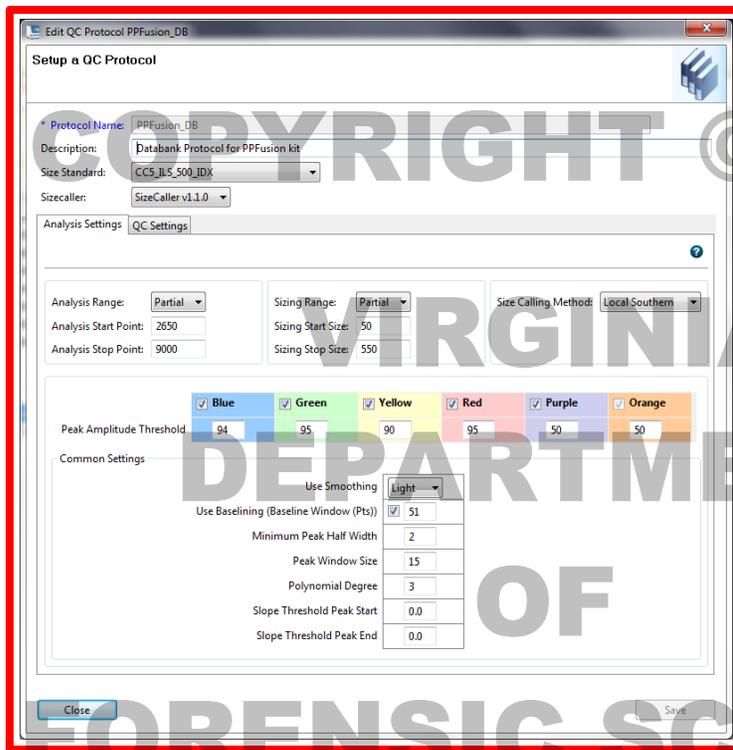
**Table 8.** Initial Data Bank file name convention settings.

9 Table 9 shows settings used in creating the Size Standard (3500xI).



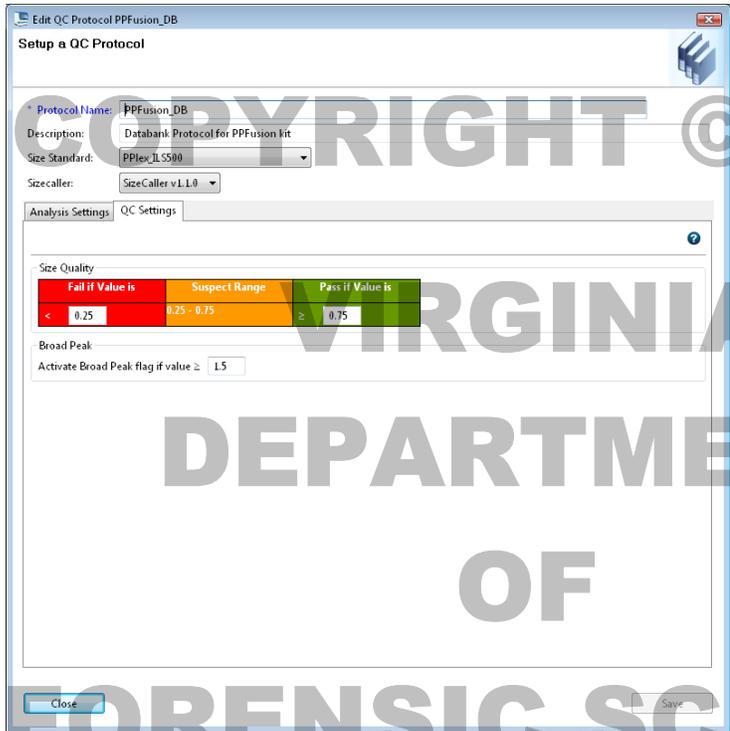
**Table 9.** Initial size standard settings.

10 Table 10 shows settings used in creating the QC protocol – Analysis settings (3500xI).



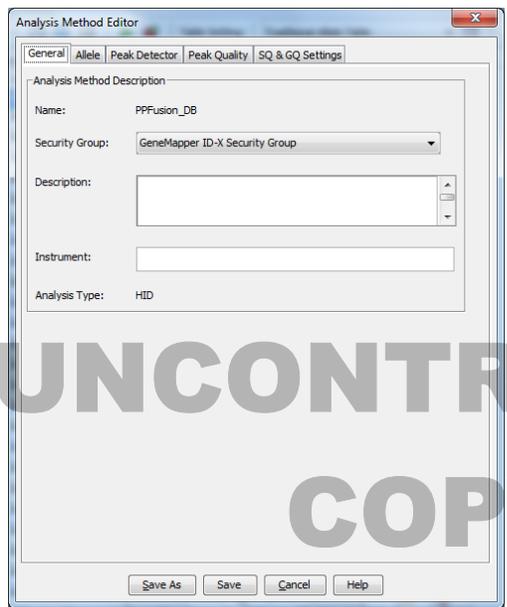
**Table 10.** Initial settings for QC protocol.

- 11 Table 11 shows settings used in creating the QC protocol – QC settings (3500x).



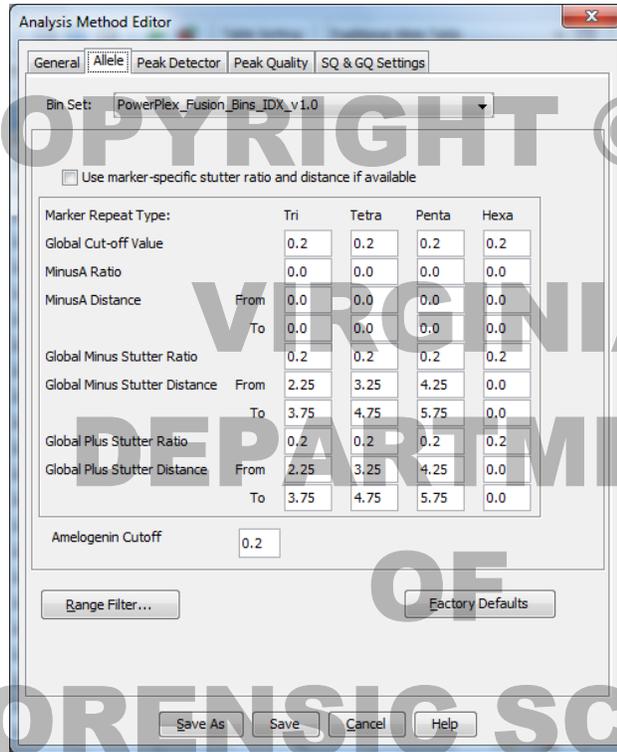
**Table 11.** Initial QC protocol settings.

- 12 Table 12 shows settings used in creating the analysis method editor – general tab (GMIDx).



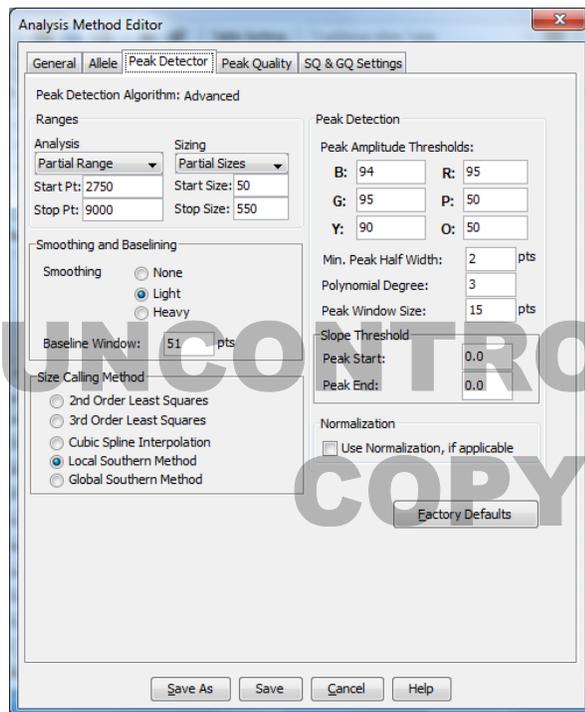
**Table 12.** Initial settings of the Analysis method editor – general tab.

13 Table 13 shows settings used in creating the analysis method editor – allele tab (GMIDx).



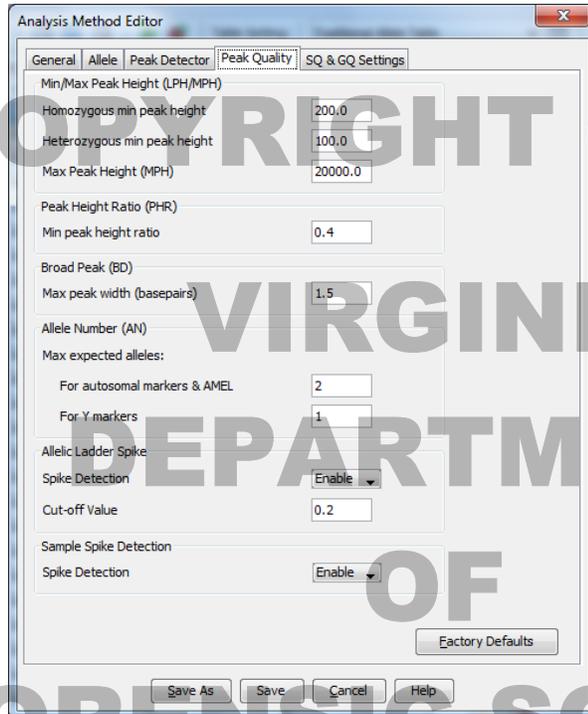
**Table 13.** Initial settings of the Analysis method editor – Allele tab.

14 Table 14 shows settings used in creating the analysis method editor – peak detector tab (GMIDx).



**Table 14.** Initial settings of the Analysis method editor – Peak Detector tab.

15 Table 15 shows settings used in creating the analysis method editor – peak quality tab (GMIDx).



**Table 15.** Initial settings of the Analysis method editor – Peak Quality tab.

16 Table 16 shows settings used in creating the analysis method editor – quality flags tab (GMIDx).



**Table 16.** Initial settings of the Analysis method editor – SQ & GQ settings tab.

17 Table 17 shows settings used in panel manager (GMIDx).

Marker Name	Dye Color	Min Size	Max Size	Control Alleles	Marker	Comments	Y Marker	Ladder Alleles
1 AMEL	Blue	80.0	92.0	%Y	9	none	<input type="checkbox"/>	9,10,11,12,13,14,15,16,17,18,19,20
2 D3S1358	Blue	93.0	150.0	17,18	4	none	<input type="checkbox"/>	9,10,11,12,13,14,14.3,15,15.3,16,16.3,17,17.3,18,18.3,19,19.3,20.3
3 D1S1656	Blue	151.0	207.0	12,13	4	none	<input type="checkbox"/>	8,9,10,11,11.3,12,13,14,15,16,17
4 D2S441	Blue	207.5	247.5	10,14	4	none	<input type="checkbox"/>	8,9,10,11,11.3,12,13,14,15,16,17
5 D10S1248	Blue	248.0	295.0	13,15	4	none	<input type="checkbox"/>	8,9,10,11,12,13,14,15,16,17,18,19
6 D13S317	Blue	295.2	360.0	9,11	4	none	<input type="checkbox"/>	5,6,7,8,9,10,11,12,13,14,15,16,17
7 Penta E	Blue	354.5	474.9	7,14	5	none	<input type="checkbox"/>	5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,22,23,24
8 D16S539	Green	73.0	129.0	9,13	4	none	<input type="checkbox"/>	4,5,6,7,8,9,10,11,12,13,14,15,16
9 D18S51	Green	129.1	215.5	16,18	4	none	<input type="checkbox"/>	7,8,9,10,10.2,11,12,13,13.2,14,15,16,17,18,19,20,21,22,23,24,25,26,27
10 D2S1338	Green	218.0	299.0	22,25	4	none	<input type="checkbox"/>	10,12,14,15,16,17,18,19,20,21,22,23,24,25,26,27,28
11 CSF1PO	Green	309.0	363.0	12	4	none	<input type="checkbox"/>	5,6,7,8,9,10,11,12,13,14,15,16
12 Penta D	Green	370.0	461.0	12,13	5	none	<input type="checkbox"/>	2,2.3,2.5,6,7,8,9,10,11,12,13,14,15,16,17
13 TH01	Yellow	69.0	118.0	6,9,3	4	none	<input type="checkbox"/>	3,4,5,6,7,8,9,9.3,10,11,13.3
14 vWA	Yellow	122.0	192.0	16,19	4	none	<input type="checkbox"/>	10,11,12,13,14,15,16,17,18,19,20,21,22,23,24
15 D21S11	Yellow	198.0	266.5	29,31.2	4	none	<input type="checkbox"/>	24,24.2,25,25.2,26,27,28,28.2,29,29.2,30,30.2,31,31.2,32,32.2,33,33.2,34,34.2,35,35.2,36,3
16 D7S820	Yellow	267.0	316.0	8,11	4	none	<input type="checkbox"/>	5,6,7,8,9,10,11,12,13,14,15,16
17 D5S818	Yellow	316.5	379.0	12	4	none	<input type="checkbox"/>	6,7,8,9,10,11,12,13,14,15,16,17,18
18 TPOX	Yellow	387.0	443.5	11	4	none	<input type="checkbox"/>	4,5,6,7,8,9,10,11,12,13,14,15,16
19 DYS391	Yellow	443.6	495.0	10	4	none	<input type="checkbox"/>	5,6,7,8,9,10,11,12,13,14,15,16
20 D8S1179	Red	72.0	131.2	14,15	4	none	<input type="checkbox"/>	7,8,9,10,11,12,13,14,15,16,17,18,19
21 D12S391	Red	131.4	192.67	18,23	4	none	<input type="checkbox"/>	14,15,16,17,17.3,18,18.3,19,20,21,22,23,24,25,26,27
22 D19S433	Red	192.3	256.5	13,14	4	none	<input type="checkbox"/>	5,2,6,2,8,9,10,11,12,12.2,13,13.2,14,14.2,15,15.2,16,16.2,17,17.2,18,18.2
23 FGA	Red	257.0	415.0	20,23	4	none	<input type="checkbox"/>	14,15,16,17,18,18.2,19,19.2,20,20.2,21,21.2,22,22.2,23,23.2,24,24.2,25,25.2,26,27,28,29,30
24 D22S1045	Red	420.0	472.0	16	3	none	<input type="checkbox"/>	7,8,9,10,11,12,13,14,15,16,17,18,19,20

Table 17. Initial panel manager settings.

18 Table 18 shows settings used for ILS set-up (GMIDx).

Size Standard Table	Size in Basepairs
1	80.0
2	95.0
3	80.0
4	100.0
5	120.0
6	140.0
7	160.0
8	180.0
9	200.0
10	225.0
11	250.0
12	275.0
13	300.0
14	325.0
15	350.0
16	375.0
17	400.0
18	425.0
19	450.0
20	475.0
21	500.0

Table 18. Initial ILS settings.